Studies into the modification of herbicide activity by chemical safeners and synergists

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STUDIES INTO THE MODIFICATION OF HERBICIDE ACTIVITY BY CHEMICAL SAFENERS AND SYNERGISTS

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CONTENTS

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ABSTRACT

Initial growth trial investigations revealed that seed dressing with the safeners naphthalic anhydride (NA) and dichlormid would partially protect Zea mays L. var. LG 11 against pre-emergence applications of the sulfonylurea herbicide chlorsulfuron. The safeners flurazole and oxabetrinil were less effective. None of the safeners protected Z. mays against the imidazolinone herbicide imazapyr. NA, dichlormid and oxabetrinil did not protect Echinochloa crus-galli against these herbicides, or to the S-triazine atrazine or the chloroacetanilide metolachlor. Some initial delay of growth was associated with all safener applications, and flurazole also reduced germination rates in both species.

Seed dressing with dichlormid enhanced the level of glutathione (GSH) in root tissue of Z. mays, and glutathione-s-transferase (GST) activity of root and shoot tissue. NA had no effect upon GSH content of the plant but did enhanced GST activity of root and shoot tissue, though to a lesser degree than dichlormid. Both safeners increased the level of acetohydroxyacid synthetase (AHAS) in Z. mays, but did not decrease the sensitivity of AHAS to chlorsulfuron. NA in vitro also had no effect upon the inhibition of AHAS by chlorsulfuron. It was concluded that the small increase in AHAS levels observed would not be sufficient to explain safener action, in the absence of any changes in the sensitivity of the target enzyme to the herbicide.

Other chemicals were investigated as potential modulators of glutathione biosynthesis in plant tissue. 2-oxo-4-thiazolidine-carboxylic acid and N-acetyl-L-cysteine were poor enhancers of GSH in Pisum sativum L. cv. Meteor leaf tissue. L-buthionine-[SR]-sulfoximine (BSO) was effective in reducing GSH levels in P. sativum and Z. mays tissue. However, BSO did not synergise the bleaching effect of paraquat in P. sativum leaf tissue or the inhibition of Z. mays growth by the thiocarbamate herbicide EPTC. BSO alone caused severe growth deformities in Z. mays, with characteristic leaf trapping and shedding of leaf cuticle. The effect of BSO upon glutathione levels in Z. mays could be partially reversed by simultaneous application of dichlormid.

Growth of cell suspension cultures of Z. mays L. var. Black Mexican Sweet were inhibited in a concentration dependent manner by NA. Fluorescein-diacetate staining of treated cells revealed no loss of viability associated with growth inhibition, except at a level where NA inhibited growth completely. 2,4-D used in the cell growth medium interacted antagonistically with NA, and could prevent growth inhibition by NA. NA was ineffective in overcoming metsulfuron-methyl inhibition of cell growth. The response of cell cultures to metsulfuron-methyl and NA was dependent upon the time of their application after subculturing.

ABBREVIATIONS

ABT	aminobenzotriazole
AHAS	acetohydroxyacid synthetase
ai.	active ingredient
ALC	N-acetyl-L-cysteine
anova	analysis of variance
BMS	Black Mexican Sweetcorn
BSO	buthionine-[S,R]-sulfoximine
CDAA	N,N-diallyl-2-dichloroacetamide
CDNB	chlorodinitrobenzene
2,4-D	2,4-dichlorophenoxyacetic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EB	Evans Blue
EC	emulsifiable concentrate
EPTC	S-ethyl dipropylthiocarbamate
FAD	flavin adenine dinucleotide
FDA	fluoroscein-diacetate
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-s-transferase
I_{50}	concentration required for 50% inhibition of enzyme activity
IAA	indoleacetic acid
ICA	2-imidazolidone-4-carboxylic acid
LD_{50}	dose required to kill 50% of test animals
MCPA	(4-chloro-2-methylphenoxy)acetic acid

MFO	mixed function oxidase
MSO	methionine sulfoximine
NA	naphthalic anhydride
OTCA	2-oxo-4-thiazolidine carboxylic acid
PAA	phenylacetic acid
PBO	piperonyl butoxide
PI	propidium iodide
ppb	parts per billion
ppm	parts per million
S.E.	standard error
2,4,6-T	(2,4,6-trichlorophenoxy)acetic acid
TPP	thiamine pyrophosphate
uv	ultra violet
v/v	volume to volume
wp	wettable powder
w/v	weight to volume
w/w	weight to weight
$\bar{x}$	average

1. INTRODUCTION: WHY MODIFY HERBICIDE ACTION

1.1 HERBICIDES AND WEED CONTROL

A weed has been defined as "any plant growing where it is not wanted" (Anderson, 1977). The presence of unwanted plants amongst a crop can lead to a reduction in both its yield and quality. Weeds compete for available resources of light, moisture and nutrients, thus restricting the growth of crop plants. They may also act as a reservoir of pests and diseases for the crop, and can hinder the process of harvesting (Roberts, 1982). The resulting yield losses may be considerable. An infestation of 36 plants per metre row of the grass weed giant foxtail (Setaria faberii) growing amongst a crop of maize resulted in a reduction of grain yield of 17% (Anderson, 1977). Auld, Menz and Tisdell (1987), reviewing estimates made for global food losses attributable to weeds have put the value at 10-20% of total production, and Chandler, Hamill and Thomas (1984) have estimated the cost of weeds in crops and pastures in the USA and Canada between 1975 and 1979 at $8,500 M.

Weeds can be controlled by mechanical, cultivational and chemical means. Prior to the introduction of chemical weed control in the 1900's, mechanical methods such as ploughing, hoeing, harrowing, seed cleaning and crop rotation techniques were employed (Roberts, 1982). These methods are still an integral part of most cropping systems. However, there is now a heavy dependence upon the use of herbicides, which have provided a good alternative to mechanical

methods alone (Anderson, 1977). An indication of the importance of chemical methods of weed control can be attained from the value of the world market for herbicides. 1984 estimates put the end-user world market value of all agrochemicals at $13.8 billion of which 43% ($5.9 billion) of product use was herbicides (Anon, 1985). In 1975, the UK alone had 461 approved herbicide products on the market (Roberts, 1982).

1.2 DEVELOPMENT OF HERBICIDES

The first recorded use of chemicals to control weeds was in 1896, when a French researcher, Bonnet, suggested the use of copper sulphate for selective weed control in cereals, after having noticed that Bordeaux Mixture used to control powdery mildew in vines, also killed the weed charlock (Sinapis arvensis). A number of other inorganic chemicals including ferrous sulphate, sulphuric acid, sodium chlorate and various arsenates were used in the late 19^{th}C and early 20^{th}C (Dodge, 1983a). The first herbicides based upon organic molecules, from which the present day herbicides have developed, were introduced in the 1930/40s. The dinitrophenol DNOC, and the phenoxyacetic acids 2,4-D and MCPA were the first, and provided good control of broad leaved weeds in cereals.

A range of herbicide compounds have since been developed, based around a number of active chemical structures, with differing modes of action. Major developments over the last 50 years have included improvements in the selectivity of herbicides for the control of

annual and perennial grass weeds in cereals, and weed control in broad leaved crops such as beans, potatoes and sugar beet (Roberts, 1982). Rates of application (Fig. 1.1) and toxicity of herbicidal compounds to non-target organisms have also been reduced over this period (Table 1.1).

1.3 LIMITATIONS AND SHORTCOMINGS OF HERBICIDES

Despite the array of herbicides now available and their range of applications, there are still limitations and challenges to be met in the chemical control of weeds.

1.3.1 Improved Selectivity

Many currently available herbicides which are effective in controlling problem weeds are either non-selective; e.g. glyphosate and paraquat, or have only marginal crop selectivity; e.g. the chloroacetanilides and thiocarbamates (Hatzios, 1989a). Chemical control of weeds in botanically related crops is still a problem, and affects many of the major world crops; e.g. wild oats (Avena fatua) in cultivated oats (Avena sativa), red rice in cultivated rice (both Oryza sativa), shattercane in grain sorghum (both Sorghum bicolor), and itch grass (Rottboelia exaltata) in maize (Zea mays) (Parker, 1983).

Fig. 1.1 Application rates of herbicides and date of introduction (after Dodge 1983a)

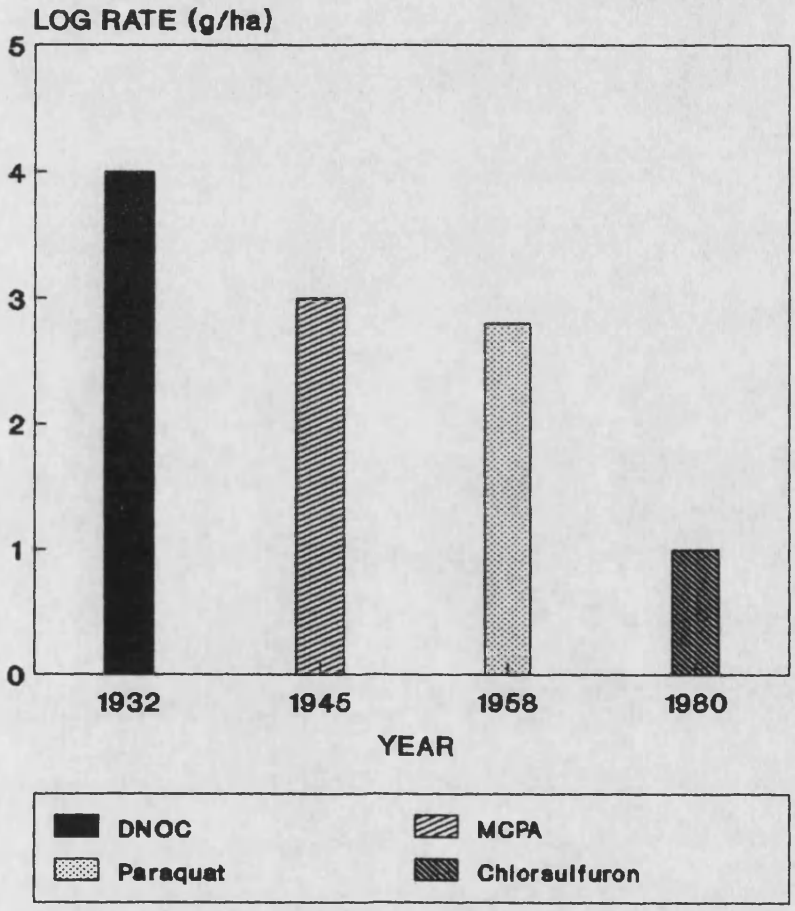


Table 1.1 Acute oral LD_{50} of selected herbicides for male rats

Date of release	Herbicide	LD_{50}
1930–40	DNOC	25–40
1940–50	2,4-D	375
	MCPA	700
1950–60	Monuron	3,600
	Atrazine	2–3,000
	Barban	1,400
	Paraquat	150
1960–70	EPTC	2,500
	Asulam	> 4,000
	Isoproturon	2–4,000
	Chlortoluron	>10,000
1970–80	Glyphosate	5,600
1980–89	Imazapyr	> 5,000
	Chlorsulfuron	5,545
	(1) * DPX A7881	>11,000
	(2) * RO 17-3664	> 5,000

Data from Worthing (1987), except *

(1) Hutchinson et al. (1987)

(2) Bocion, Muehlethaler and Winternitz (1987)

1.3.2 Development of Resistant Weeds

Although less rapid than the development of resistance by target organisms to other pesticides, several instances of weed resistance to herbicides have been recorded (Le Baron and Gressel, 1982). These have occurred throughout North America and Europe, and in areas in the Middle and Far East, and Australia and New Zealand, to a range of herbicides (Gressel, 1987). Examples include resistance to oxidant generating herbicides such as paraquat by Conyza spp. in Hungary, Japan and Egypt, and annual meadow grass (Poa annua) in England (Gressel, 1987), and to photosystem II inhibitors such as simazine by groundsel (Sencio vulgaris), annual meadow grass (Poa annua) and American willowherb (E. ciliatum) (Putwain, 1982). More recently, resistance has been reported in blackgrass (Alopecurus myosuroides) to some phenylureas and sulfonylureas (Moss and Cussans, 1985), and to the phenoxy herbicide mecoprop in chickweed (Stellaria media) (Lutman and Snow, 1987). Cross resistance to other herbicides has been identified in some of these weed populations (Lutman and Snow, 1987; Polos et al., 1987).

1.3.3 Changes in Crops and Cropping Systems

Changes in cropping systems, and crops grown may require concomitant changes in weed management policies and the herbicides used.

Reduced cultivations and rotations have represented significant changes in cultural practices in U.K. agriculture in the last 20 years. An increase in the growth of winter cereals and the cultivation of oilseed rape (Brassica napus var. Oleifra) as a major breakcrop has also occurred requiring the adoption of new crop protection methods (Anon, 1987a). Alternative crops under review at the moment include sunflowers (Helianthus annuus), lupins (Lupinus albus), soyabeans (Glycine max), evening primrose (Oenothera spp.) and linseed and flax (both Linum usitatissimum). There are as yet few herbicides registered for use in linseed and flax in the U.K., and those which are available are limited both in selectivity and the range of weeds controlled (Dover, Roebuck and Wolley, 1987). Hence, there is a continuing need for new compounds for new crops.

1.3.4 Environmental Considerations

There is an increasing public awareness of the potential hazards of pesticide usage through residues in food and damage to the environment (Conway, Gilbert and Pretty, 1988). This has been reflected in changes in legislation in the U.K. controlling the sale, supply, distribution, use and marketing of pesticides, and the setting of residue limits. The Food and Environment Protection Act (FEPA) (1985) and Control of Pesticides Regulations (CPR) (1986) were recently passed to provide statutory control of these factors in place of the previous voluntary schemes, i.e. Agricultural Chemicals Approvals Scheme (ACAS) and Pesticides

Safety Precautions Scheme (PSPS) (Anon, 1986). There will be increasing pressure on agrochemical companies to produce more "environmentally friendly" products, which must include reduced toxicity to non-target organisms, lower inputs, and reduced residues. There is already a noticeable involvement of agrochemical companies in environmental and wildlife conservation projects (Anon, 1987b) which reflects this, and at least one company, Schering Agriculture, has adopted a "Green Science" logo to highlight the responsibility it feels towards the environment in its crop protection business!

1.4 METHODS FOR DEVELOPING CHEMICAL WEED CONTROL

There are several approaches which are available to agrochemical companies to meet these challenges.

1.4.1 Improve Application Methods and Formulation of Products

Improvements in application methods and formulation can enhance the selectivity of existing products, improve efficiency of use, and allow lower rates to be applied.

Adjuvants which act as wetters, stickers, spreaders etc. in post-emergence herbicide applications, are used to enhance the biological activity of the products through enhanced uptake and retention on leaf surfaces (Wills and McWhorter, 1983). Polymers such as α-cellulose, lignin and starch in granular soil applied

herbicides allow controlled release of herbicides, restricting leaching from the weed seed zone to crop roots, and hence enhancing selectivity (Riggle and Penner, 1989).

Timing and placement of herbicides by spot application or band spraying can also enhance selectivity (Roberts, 1982). Improved droplet-generation in sprays towards a narrower size spectrum, and electrostatic charging of droplets can increase spray retention, distribution on plants, and restrict spray drift (Corty, 1983). The development of weed wipers in recent years has provided another means of selective application and improved efficiency (Corty, 1983; Roberts, 1982).

1.4.2 Develop Novel Herbicide Compounds

Novel compounds are continuing to be released onto the market by agrochemical companies, which have improved characteristics such as reduced mammalian toxicity, improved selectivity and reduced application rates.

The imidazolinones and sulfonylureas are two recently introduced herbicide groups and reasonably successful commercial compounds (Hawkes, Howard and Pontin, 1989). Both were developed independently through random screening methods. They are active at very low application rates (g/ha), have low mammalian toxicity (LD_{50} male rats > 5000 mg/kg) and contain a number of different active structures within the group, with a range of characteristics

with regard to selectivity and activity. Both groups have the same site of action in plants; inhibition of the enzyme acetohydroxy-acid synthetase (AHAS) which is involved in the biosynthesis of the branched chain amino acids - valine, leucine and isoleucine. This is a novel target site compared to other previous herbicidal groups.

However, the expense and time invested in producing new herbicides is great, and the number of compounds being released each year is diminishing (Roberts, 1982). Development time is a minimum of six years for early screening, toxicological studies, trials etc. and initial development. The cost of developing a herbicide is rising and has been estimated at over $20 M (Parry, 1989), with no return seen on this money for at least 10 years. Research and development costs are rising faster than the rate of market expansion, and there are also increasing costs of registration to be met. These have led to the merging of several agrochemical companies in recent years (Parry, 1989).

1.4.3 Genetic Manipulation of Crops to Confer Herbicide Tolerance

The use of cheap, "environmentally safe" herbicides which possess only marginal or no selectivity, can be extended by selecting for, or introducing into crops, genes which confer resistance or enhanced tolerance to these compounds (Gressel, Ezra and Jain, 1982).

Gene transfer, cell selection and standard plant breeding techniques have been used successfully to create crops and other plants which are tolerant to several major commercial herbicides (Gressel, 1989). These include: glyphosate resistance in tobacco (Nicotiana tabacum), petunia (Petunia hybrida) and tomato (Lycopersicon esculentum); phosphinothricin tolerance in alfalfa (Medicago sativa) tomato, oilseed rape, and potato (Solanum tuberosum); sulfonylurea tolerance in tobacco and Arabidopsis thaliana; imidazolinone tolerance in maize; atrazine tolerance in oilseed rape; and bromoxynil tolerance in tobacco and tomato (Fraley et al., 1987). These examples include; changes in target site proteins, e.g. 5-enolpyruvylshikimate-3-phosphate synthetase (EPSPS) in glyphosate tolerance and acetodroxyacid synthetase (AHAS) in imidazolinone and sylfonylurea tolerance, enhanced production of target site proteins e.g. EPSPS in glyphosate resistance; and enhancement of herbicide metabolism, e.g. bromoxynil tolerance by introduction of a nitrilase enzyme, or phosphinothricin acetyl transferase for phosphinothricin tolerance (Gressel, 1989).

Atrazine tolerant varieties of oilseed rape are already available (Souza Machado and Hume, 1987) and other commercial crops will probably be produced in the early 1990s (Fraley et al., 1987).

However, there are some problems with this approach, as herbicide tolerance can be associated with undesirable agronomic traits. Triazine tolerant oilseed rape exhibits reduced competitive vigour,

oil yield and content, and delayed maturity compared with non-tolerant varieties (Marshall, 1987). These changes may be due to reduced photosynthetic rates associated with changes in the herbicide target site protein in the chloroplast (Marshall, 1987). There is also the risk of the introduction of herbicide tolerance into closely related weed species. Selective weed control of wild oats in cultivated oats using a diclofop-methyl tolerant cultivar may not be feasible because of the risk of inter-specific crossing (Warkentin et al., 1988).

For agrochemical companies to make full use of this approach they will have to produce engineered crop/herbicide packages for the market. In this respect, several companies have recently been purchasing seed companies (Marshall, 1987). Costs associated with registering existing herbicides for use in new crops may deter some development, as will questions of "patenting" tolerant cultivars, and controls on the release of genetically engineered organisms into the environment.

1.4.4 Chemical Manipulation of Crop and Weed Tolerance to Herbicides

The activity of herbicides in plants can be synergised or antagonised by the application of other chemicals (Hatzios and Penner, 1985). Antagonism of herbicide action can be used to protect crops from herbicides with little or no selective action. Similarly, synergism of herbicide activity can help to control

problem weeds and reduce herbicide application rates. This is an approach which has already been used by agrochemical companies to extend the use of existing herbicides. The development, use and mechanisms of action of chemical herbicide "safeners" and "synergists" are reviewed in Chapter 2.

2. HERBICIDE SAFENERS AND SYNERGISTS: LITERATURE REVIEW

2.1 TERMINOLOGY

A number of different terms have been used, often interchangeably in the literature, to describe chemicals which reduce the toxicity of herbicides to plants. These include; herbicide antidote, herbicide adsorbant, antagonist, herbicide safener, crop protectant, agriregulator and herbicide modifier (Hatzios,1989a).

The use of these different terms has been discussed previously (Codd, 1988; Hatzios, 1989a; Parker, 1983). The term "herbicide antidote", introduced by Hoffmann (1962) to describe chemical agents which protect crop plants from herbicide injury, has received some criticism. This is in part due to the confusion which might arise from the belief that "herbicide antidotes" are a pharmaceutical product for the counteraction of herbicide toxicity in humans, analogous to other "antidotes" for drugs and poisons. It has also been found to be an inappropriate term because unlike pharmaceutical "antidotes" which reverse the toxic effect of chemicals after they have been applied, "herbicide antidotes" prevent herbicide damage rather than reversing it, and consequently have to be applied prior to, or at the same time as, the herbicides (Hatzios, 1983c; Parker, 1983). Consequently, this term has generally been replaced by the word safener. Parker (1983) objected to this on the grounds that there is no verb "to safen" in the English language. However, the term safener has been adopted by

workers in this field. Codd (1988) argued that "safener" met the main criteria required for a new word to be accepted in a dictionary. Herbicide adsorbent has been used to describe chemicals such as charcoal or lignin by-products which act externally to the plants, preventing herbicides reaching the plant surface (Blair, Parker and Kasasian, 1976; Hatzios, 1983c; Hoagland, 1989), as opposed to safeners or antidotes which act internally. Crop protectant has been used to include both safeners and adsorbents (Hatzios, 1983c).

Herbicide antagonist has been used synonymously with herbicide safener (Hatzios, 1989a) but Codd (1988) suggested that this should perhaps be restricted to use with the subgroup of herbicide safeners which are recognised to have opposing physiological effects to herbicides, as the general meaning of antagonist suggests.

Some confusion also surrounds the use of "herbicide synergist" in the literature. There is good agreement about the term "synergism" e.g. "any combination which has greater effect together than the sum of the components" (Gressel and Shaaltiel, 1988); "substantially more than additive toxic or pharmacological action of two substances used together" (Metcalf, 1967). However, even here some problems arise. Gressel and Shaaltiel (1988) reporting on a survey of recent patents found examples of herbicide "synergism" where two herbicides together controlled more weed species than each separately. This overlap in the range of weeds controlled by

each herbicide, they believed should be better described as "complementarity", as it might or might not be due to metabolic synergy.

The above definitions of synergism could be used to classify adjuvants such as wetting agents, stickers, spreaders, petroleum and crop oils, as synergists. These are used in herbicide formulations to enhance the uptake of herbicides through better retention and distribution over the plant surface (Wills and McWhorter, 1983). Hatzios and Penner (1985) included these types of adjuvants in examples of synergistic interactions between herbicides and other agrochemicals. However, the term herbicide synergist has tended to be applied to chemicals which have an internal metabolic effect in enhancing herbicide action (Cabanne et al., 1987; Ezra, Dekker and Stephenson, 1985; Gressel and Shaaltiel, 1988; Lamoureux and Rusness, 1986b) rather than just an external physical effect in enhancing herbicide uptake.

In this thesis, the term herbicide synergist will refer to chemicals which enhance the action of herbicides in a more than additive way and act primarily at an internal site. Gressel and Shaaltiel (1988) have suggested two subdivisions within the term synergist, one for combinations of active herbicides, and the other for herbicide/adjuvant combinations where one of the components is non phytotoxic.

2.2 HISTORY, DEVELOPMENT AND USES

2.2.1 Herbicide Safeners

The discovery and development of herbicide safeners began with a chance observation by Hoffmann in 1947 that 2,4,6-T, a structural analogue of the herbicide 2,4-D, protected tomato plants from the toxic effects of 2,4-D fumes in a greenhouse (Hoffmann, 1953). Further investigation revealed that 2,4-D, 2,4,6-T and MCPA applied as foliar sprays could protect wheat from damage by the carbamate herbicide barban (Hoffmann et al., 1960; Hoffmann, 1978).

Hoffmann realised the benefits which might be achieved if this type of antagonistic interaction could be used to protect crop plants from herbicides which gave good weed control, but had only marginal selectivity. Twenty-one years of research followed Hoffmann's original observation before the first commercially available safener was produced (Hoffmann, 1978). 2,4-D, 2,4,6-T and MCPA could not be used as foliar sprays on wheat as they also antagonised the effect of barban on wild oats. Attempts to safen wheat selectively using these compounds as seed dressings failed as they proved to be too toxic when applied in this manner (Hoffmann, 1978). Screening of compounds that caused 2,4,-D like symptoms on tomatoes, but were safe as a seed dressing on grass crops, produced S-449 as an effective chemical safener of barban injury in wheat (Hoffmann, 1962). This was followed in 1969 by the introduction of 1,8-naphthalic anhydride (NA), the first commercially available

safener, which provided effective protection against the thiocarbamate herbicide EPTC in maize (Hoffmann, 1969). This was patented and sold by the Gulf Oil Chemical Company in 1971 (Hoffmann, 1971).

Research by other chemical companies quickly produced several commercial safeners which were based on different chemical structures. Researchers at Stauffer Chemical Company found that dichloroacetamide compounds could protect maize from thiocarbamate herbicides, and dichlormid (N,N-diallyl-2,2-dichloroacetamide also referred to as R 25788) was patented by Stauffer for this purpose in 1972 (Pallos, Brokke and Arneklev, 1977; Pallos et al., 1978). Unlike most other safeners, dichlormid was found to be effective when soil applied with EPTC, as it provided no protection to grass weeds (Stephenson and Chang, 1978).

In 1974, workers at Ciba-Geigy Corporation found that oxime ether compounds provided protection against acetanilide herbicides such as metolachlor to grain sorghum. Cyometrinil (α-[(cyanomethoxy) imino]-benzene acetonitrile or CGA 43089) was introduced in 1978 (Martin, 1978) as a seed dressing for sorghum against acetanilide herbicides. Oxabetrinil (α[((1,3-dioxolan-2-yl)methoxy)-imino] benzeneacetonitrile also referred to as CGA 92194) a chemical analogue of cyometrinil, was introduced in 1982 (Dill et al., 1982). This provided similar protection to cyometrinil, but was better tolerated by the crop (Turner et al., 1982; Chang and merkle, 1982). A third oxime ether CGA 133205 has recently been

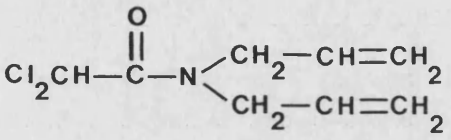
introduced for the same purpose (Dill, 1986; Helseth and Dill, 1986).

Monsanto Chemical Company produced flurazole (benzyl 2-chloro-4-(trifluoromethyl) thiazole-5-carboxylate, also referred to as MON 4606) in 1980, which used as a seed dressing protects sorghum from alachlor injury (Howe and Lee, 1980; Schafer, Brinker and Radke, 1981). This represented a third type of safening compound, a derivative of 2,4-disubstituted 5-thiazolecarboxylic acid.

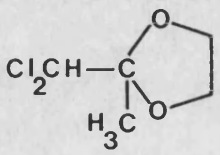
A fourth and fifth group of safener compounds have recently been introduced. Fenclorim (CGA 123407) is a 1983 Ciba-Geigy compound for protection against pretilachlor in rice (Quadranti and Ebner, 1984), and is an example of a substituted phenyl-pyrimidine. MG 191, introduced by Nitrokemia in 1986 is a safener for thiocarbamates in maize (Dutka and Kömives, 1987), and is a dichloromethyl-dioxolan compound.

Other products are under development, such as CGA 154281; which is a new safener for metolachlor in maize (Peek et al., 1988). Hatzios (1989a) lists 14 patented safener products excluding NA and S-449. Numerous examples of antagonistic interactions between agrochemicals and herbicides have been reported in the literature (Hatzios and Penner, 1985) and could provide leads for further classes of safeners in the future.

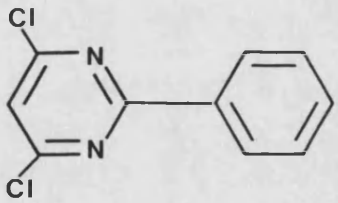
Fig. 2.1 Examples of safener compounds



DICHLORMID



MG 191



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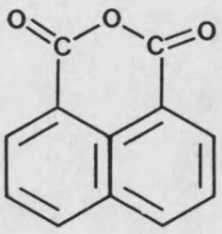
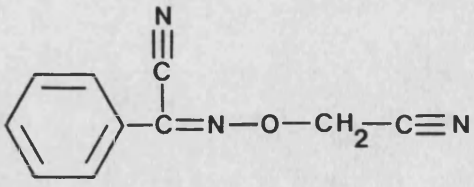
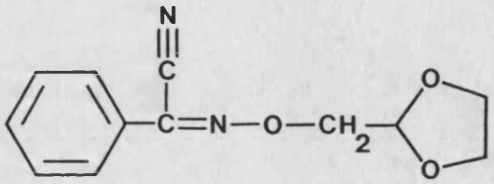


NAPHTHALIC ANHYDRIDE

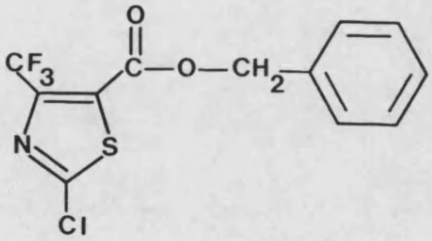


CYOMETRINIL

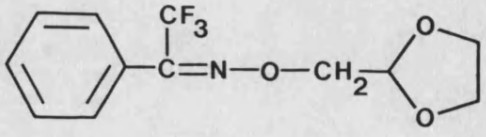
Fig. 2.1 continued



OXABETRINIL



FLURAZOLE



CGA 113205

Field usage of safeners has been limited by the botanical and chemical specificity of the compounds. Hatzios (1989a) lists 10 commercially available safeners used in a total of 14 formulations. These products are limited to use in the cereal crops, maize, wheat, sorghum and rice, with thiocarbamate and chloroacetanilide herbicides. Safening against herbicides in broad leaved crops has proved elusive (Parker, 1983; Hatzios, 1983c), as has protection against photosynthetic inhibitor herbicides such as the s-triazines and phenylureas, and bleaching herbicides such as the bipyridiums. However, Devlin and Koszanski (1987 a, b) have recently reported that dichlormid provides some degree of protection to maize against two new herbicides, clomazone and SC-0744, which exhibit bleaching activity. Safening against some other classes of herbicide, e.g. NA/dichlormid against sulfonylureas in maize, is only partial (Hatzios, 1983c) and hence of little practical value.

Field performance of some safeners has proved to be inconsistent compared with greenhouse trials. Turner et al. (1982) reported inconsistent protection of sorghum with cyometrinil in the field, and adverse effects upon seed germination and establishment. Codd (1988) reported similar findings for the protection of oats against diclofop-methyl in the field. Temperature, light, soil moisture and soil type have been found to affect field performance of safeners (Hatzios, 1983c). Mechanical loss of chemials used as a seed dressing during the planting process, may account for some of the erratic safener performance in the field (Codd, 1988).

Herbicide safener development and use has been the subject of a number of reviews (Blair, Parker and Kasasian, 1976; Gray et al., 1982; Hatzios, 1983c, 1989a; Parker, 1983; Fedtke, 1985).

2.2.2 Herbicide Synergists

Unlike herbicide safeners, few if any products have been developed specifically as herbicide synergists. Examples of synergists do occur for insecticides, e.g. piperonyl butoxide and S421 are marketed as synergists for pyrethrins and related insecticides (Hartley and Kidd, 1989).

Hatzios and Penner (1985) list many examples of synergistic interactions between herbicides and other agrochemicals, i.e. fungicides, herbicides, insecticides, fertilizers and adjuvants. One of the earliest examples of these was the synergism of propanil in rice by carbamate and organophosphate insecticides (Frear and Still, 1968; Matsunaka, 1969). A survey of the herbicide products in Hartley and Kidd (1988) and Worthing (1987) with cross reference to Hatzios and Penner (1985), reveals that a number of herbicide formulations contain synergistic combinations of herbicide/herbicide or herbicide/adjuvant. Table 2.1 summarises these synergistic combinations.

Most of the examples cited in Table 2.1 are for non-selective herbicides, e.g. amitrole, diuron, paraquat, or herbicides used at levels where they are total herbicides e.g. simazine. The example

Table 2.1 Synergistic combinations of herbicide/herbicide and herbicide/adjuvant used in commercial herbicide formulations

Herbicide	Herbicide/ adjuvant	Product (Company)	Weed	Reference
Amitrole	Ammonium thiocyanate	Radoxone TL (SOPRA) Weedazol TL (Union carbide)	Agropyron repens	Donnaley and Ries (1964)
Amitrole	Paraquat	Groundhog (ICI)		
Simazine	Paraquat	Gramazine (ICI) Terraklene (SOPRA)	Agropyron repens	Putnam and Ries (1965,1967)
Diuron	Paraquat	Dexuron (Chipman) Totacol (SOPRA)		
Simetryn	MCPA	Grakill (Hokko Chemical)	Echinochloa crus-galli	Hagimoto and Yoshikawa (1972)
Simetryn	Thiobencarb	Saturn (Hokko Chemical)		
Alachlor	Atrazine	Lasso (atrazine) (Monsanto) Rambo (Siapa)	Echinochloa crus-galli	Akobundu, Sweet and Duke, (1975)
Atrazine	Tridiphane	Tandem (Dow)	Panicum milaceum	Ezra, Dekker and Stephenson (1985)
			Setaria faberi	Zorner (1983)

of atrazine with the herbicide tridiphane is one where selective control of problem weed grasses such as Panicum milaceum (Proso Millet) and Setaria faberi (giant foxtail) in maize is possible (Ezra, Dekker and Stephenson, 1985; Zorner, 1983). Tridiphane has also been reported to synergise the action of alachlor and EPTC on Panicum milaceum (Dekker, 1984; Ezra, Dekker and Stephenson, 1985) both of which are selective herbicides for use in maize.

Other herbicide/herbicide and herbicide/adjuvant combinations have been reported to interact synergistically. Glyphosate/imidazolinone mixtures caused 97% necrosis in eight test weed species compared to 0% and 25% respectively for the herbicides applied alone (Bocion, 1987). Mixed function oxidase (MFO) inhibitors such as aminobenzotriazole (ABT) and piperonyl butoxide (PBO) synergised the activity of chlorotoluron and isoproturon in wheat (Gaillardon et al., 1985). ABT has also been used to synergise the activity of these herbicides in resistant populations of blackgrass (Alopecurus myosuroides) (Kemp and Caseley, 1987). Gressel and Shaaltiel (1988) have reported synergism of active-oxygen generating herbicides such as atrazine and paraquat with copper and zinc chelators, e.g. di-2-ethyl-hexyl-phosphoric acid. None of the last examples may be viable as commercial products, but the use of these types of adjuvants to synergise herbicide activity may produce useful leads for the development of commercial products in the future.

The use of chemicals as herbicide synergists will always be more difficult than using them as safeners, because of the problem of

selectivity. Safener selectivity can be ensured by using the compounds as a seed dressing as is the case with many of the commercially available products (Section 2.2.1). However, it is much more difficult to apply a synergist plus herbicide selectively to weeds and not to crop plants. If this was possible, then a non-selective herbicide could be used in any case. The use of compounds such as ABT to control chlortoluron/isoproturon resistant blackgrass in wheat would seem to be impractical because of the problem of synergism to the crop. However, a combined application of synergist and herbicide to crop and weeds can work, as has been reported for tridiphane and atrazine in maize. Here the synergist acted selectively in the weed plants and not in the crop (Boydston and Slife , 1986). It is also interesting to note that most of the cited synergistic herbicide combinations are for total herbicides (non-selective), where there is already good provision for weed control with compounds such as glyphosate, paraquat and imazapyr.

2.3 MECHANISMS OF SAFENER ACTION

2.3.1 An Overview

Although chemical herbicide safeners have been used for nearly 20 years the mechanism(s) by which they exert their protective action are still far from clear.

Several reviews have included discussion of the mechanisms of safener action (Blair et al., 1976; Fedtke, 1985; Hatzios, 1983c,

1989b; Parker, 1983; Stephenson and Ezra, 1982; Wakabayshi and Matsunaka, 1982).

Hatzios (1983c) summarised the main mechanisms which have been proposed as;

1. Safener induced reduction of herbicide uptake and/or translocation within the protected plant.

2. Counteraction of herbicide phytotoxicity through competitive inhibition at some common site within the plant.

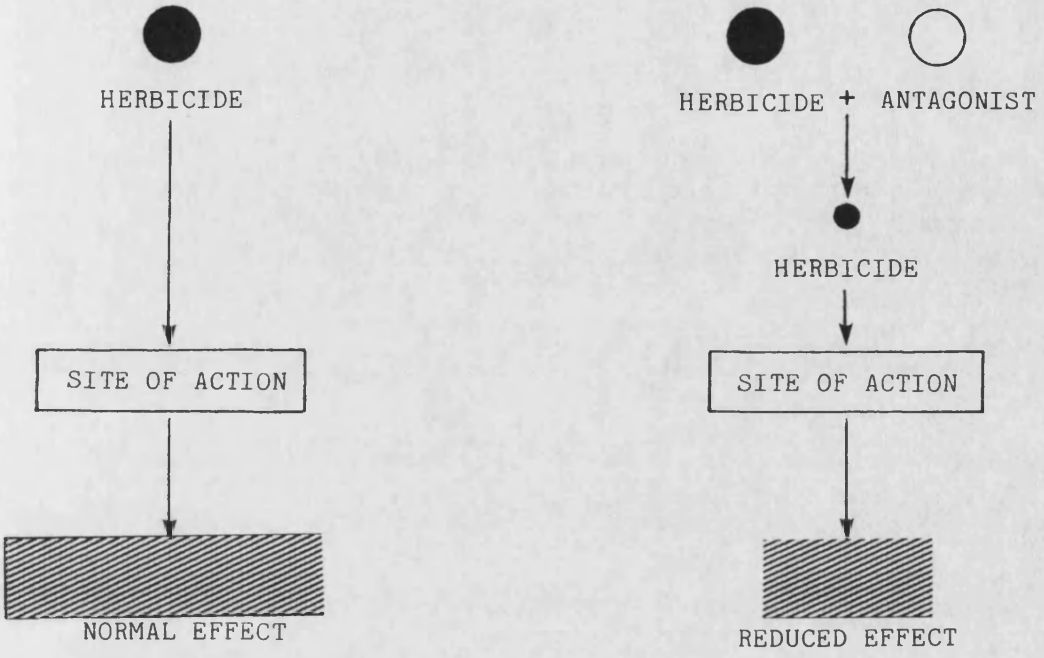
3. Safener induced stimulation of herbicide degradation by the protected plant.

4. Any combination of 1-3.

A slightly modified and more detailed form of these mechanisms (Fig. 2.2) has been proposed by Hatzios and Penner (1985) to describe herbicide antagonism in general. This has subsequently been applied to safeners (Hatzios, 1989 b). Schemes 1 and 2 (Fig. 2.2) have safeners acting as "bioregulators" - influencing the amount of herbicide at the target site in active form. Schemes 3 and 4 depict safeners as "antagonists" of herbicide action, either at a common site of action or by acting at separate sites.

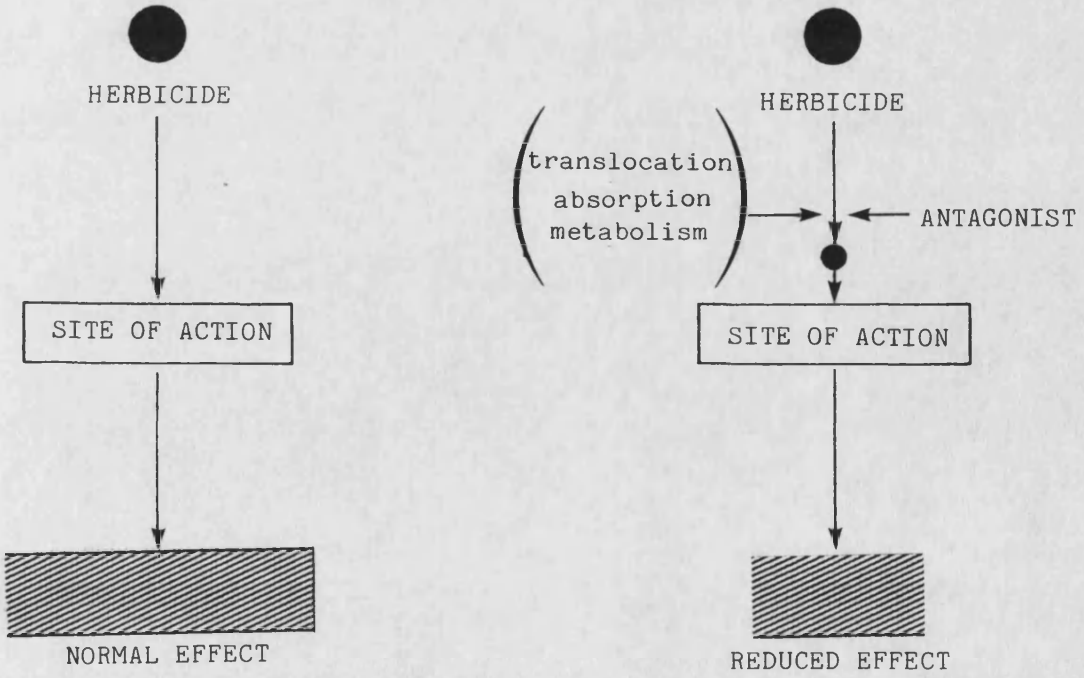
Fig. 2.2 Proposed mechanisms of safener action (after Hatzios and Penner, 1985)

1. CHEMICAL ANTAGONISM



(Safener forms inactive chemical complex with herbicide)

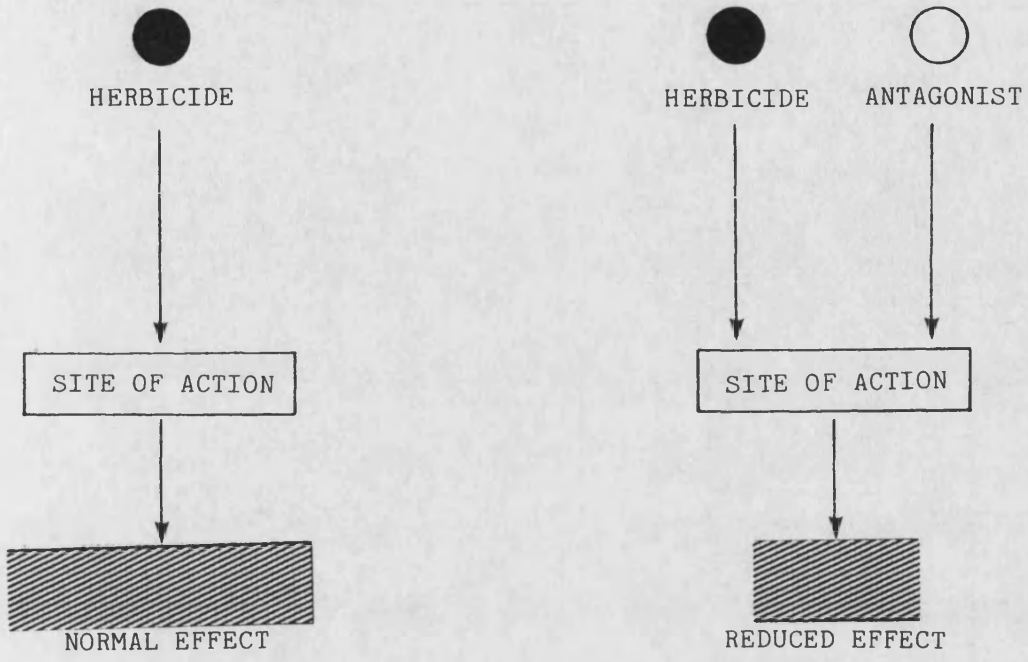
2. BIOCHEMICAL ANTAGONISM



(Safener induces changes in herbicide penetration, translocation or metabolism)

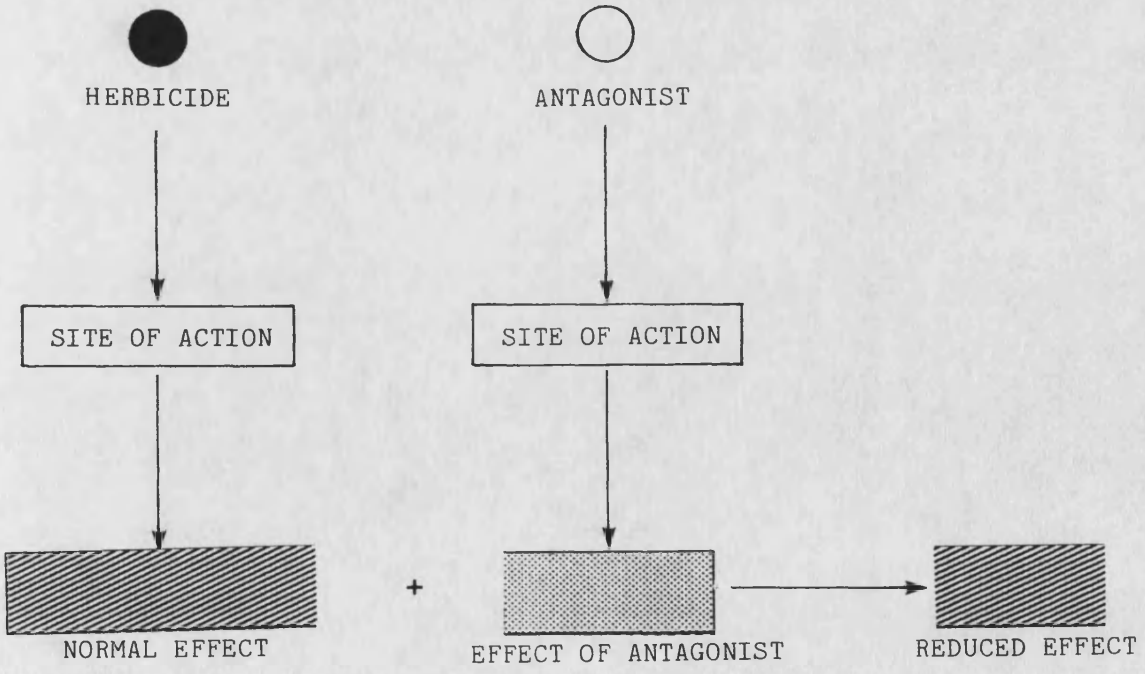
Fig. 2.2 continued

3. COMPETITIVE ANTAGONISM



(Safener competes with the herbicide at a common site of action)

4. PHYSIOLOGICAL ANTAGONISM



(Safener acts at a separate site but induces changes which counteract the effect of the herbicide)

Wakabayshi and Matsunaka (1982) included a "compensation" type of safener action which does not fit directly into this scheme. An example of this was the relief of herbidical symptoms of the Hill reaction inhibitors, phenylureas and s-triazines, by the addition of sugars. It should not be overlooked however, that a reduction of herbicide metabolism could produce a safening response for herbicide which are activated by metabolism (Hatzios and Penner, 1982), although no such safening response has been reported. Of the mechanisms suggested, enhancement of herbicide metabolism and competitive antagonism of herbicides and safeners at a common site of action, have been most favoured as explanations of safener action. More detailed evidence for the involvement of these mechanisms in safener action are discussed in the following sections.

2.3.2 Alteration of Herbicide Uptake and Translocation

Herbicide safeners and the thiocarbamate and chloroacetanilide herbicides are taken up mainly through the coleoptile of grass crops (Hatzios, 1989b). Hence, the possibility that safeners might act by reducing the uptake and/or translocation of herbicides to their active sites has been investigated.

There is little evidence in the literature to support the hypothesis that safeners reduce herbicide uptake. Ezra, Krochmal and Gressel (1982) found that dichlormid inhibited the uptake of ^{14}C EPTC into maize cells in suspension culture when they were

applied simultaneously. Inhibition was competitive, and uptake was not via an active transport system. It was suggested that the similarity in chemical structure of EPTC and dichlormid might explain the competition for uptake into the cells. However, CDAA which is also structurally similar to dichlormid had much less effect upon EPTC uptake. GA_3, NA, and cyometrinil either had no effect, or enhanced uptake of the herbicide.

Most other reports have indicated that safeners enhance the uptake of herbicides, or have little or no effect. Murphy (1972) found that NA enhanced ^{14}C EPTC uptake into safened maize plants. Rubin, Kirino and Casida (1985) reported increased alachlor uptake into sorghum plants with N-(4-chlorophenyl) maleamic acid (CPMA) or flurazole treatment, although by seven days both safeners reduced the level of herbicide in the shoot compared to controls. However, in these reports herbicide uptake was expressed as the total per plant which did not take into account the extra growth of safened plants compared to herbicide treatments alone. Chang, Stephenson and Bandeen (1974) found no change in EPTC uptake and distribution in maize with dichlormid treatment, when expressed per unit weight of plant material, but the safener did increase the total amount of EPTC taken up per plant. Similarly, Jackson, Yopp and Kapusta (1986) reported no effect of flurazole on acetochlor absorption and distribution in sorghum.

Experiments of shorter duration have indicated safener induced enhancement of herbicide uptake. Oxabetrinil, cyometrinil,

flurazole, NA, and dichlormid pretreatments enhanced metolachlor uptake into sorghum shoots (Zama and Hatzios,1986a; Fuerst and Gronwald, 1986). CDAA pretreatments enhanced subsequent CDAA uptake into maize roots (Ezra et al., 1985). Zama and Hatzios (1987) using isolated leaf mesophyll protoplasts of sorghum found that simultaneous application of oxabetrinil enhanced ^{14}C metolachlor uptake, and that stimulation was dependent on the safener concentration.

These changes in herbicide uptake and movement with safener treatment do not indicate that this is an important mechanism of safener action.

2.3.3 Enhancement of Herbicide Detoxification

a) Glutathione/glutathione-s-transferase system

A number of groups of herbicides are detoxified in plants via conjugation with the tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH). These include the thiocarbamates, chloroacetanilides and s-triazines (Hatzios and Penner, 1982; Lamoureux and Rusness, 1986a). Homoglutathione (glutamylcysteinyl-β-alanine) replaces glutathione in some plant species (Breaux, Patanella and Sanders, 1987; Rennenberg, 1982).

The sulphydryl (SH) group is the most important structural feature in the interaction of glutathione/homoglutathione with xenobiotics.

Conjugation of thiocarbamates occurs after oxidation to their corresponding sulfoxides or sulfones (Dutka and Kömives, 1983). Chloroacetanilides conjugate directly with GSH, and do not require any activation step (Leavitt and Penner, 1979). The conjugation reaction is catalysed by glutathione-s-transferases (GST), but can also occur non-enzymatically at a low rate under physiological conditions (Ezra et al., 1985; Gronwald et al., 1987; Leavitt and Penner, 1979). Investigation of plant GSTs has revealed that a number of isoenzymes occur in plants, and that these differ in their substrate specifities (Edwards and Owen, 1986a, 1987; Mozer, Tiemeier and Jaworski, 1983). Activity is associated with both cytostolic and microsomal fractions of plant extracts (Edwards and Owen, 1987; Kömives, Kömives and Dutka, 1985).

Glutathione conjugation is the primary detoxification step for chloroacetanilides and thiocarbamates (Hatzios and Penner, 1982), although in the case of the herbicide tridiphane, GSH conjugation has been found to be an activation step (Lamoureux and Rusness, 1986b). GSH conjugates are further metabolised in plants, often to cysteine and thioacetic acid conjugates (Lamoureux and Rusness, 1986a) (Fig. 2.3). Some safeners, in particular the dichloroacetamides, have been found to elevate GSH levels and enhance GST activity in treated tissue. Lay, Hubbel and Casida (1975) reported that dichlormid and the related dichloromacetamide R 29148 enhanced GSH content of maize roots by a factor of two, and GST activity by a factor of nine. Lay and Casida (1976, 1978) used dichlormid and a series of related compounds to show that

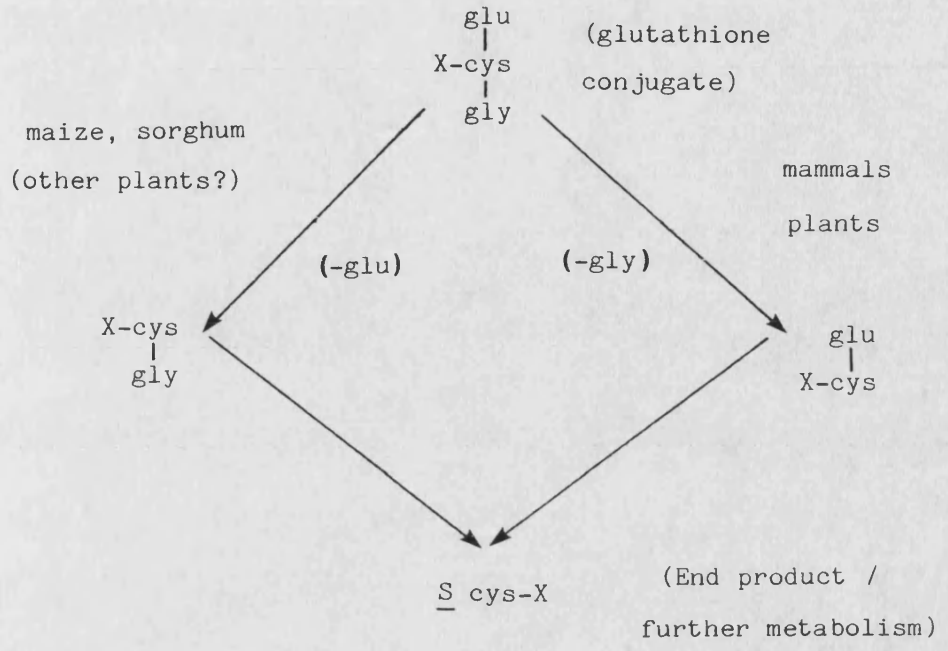
Fig. 2.3 Metabolism of glutathione conjugates in higher plants (after Lamoureux and Rusness, 1986a; Bakke, 1986)

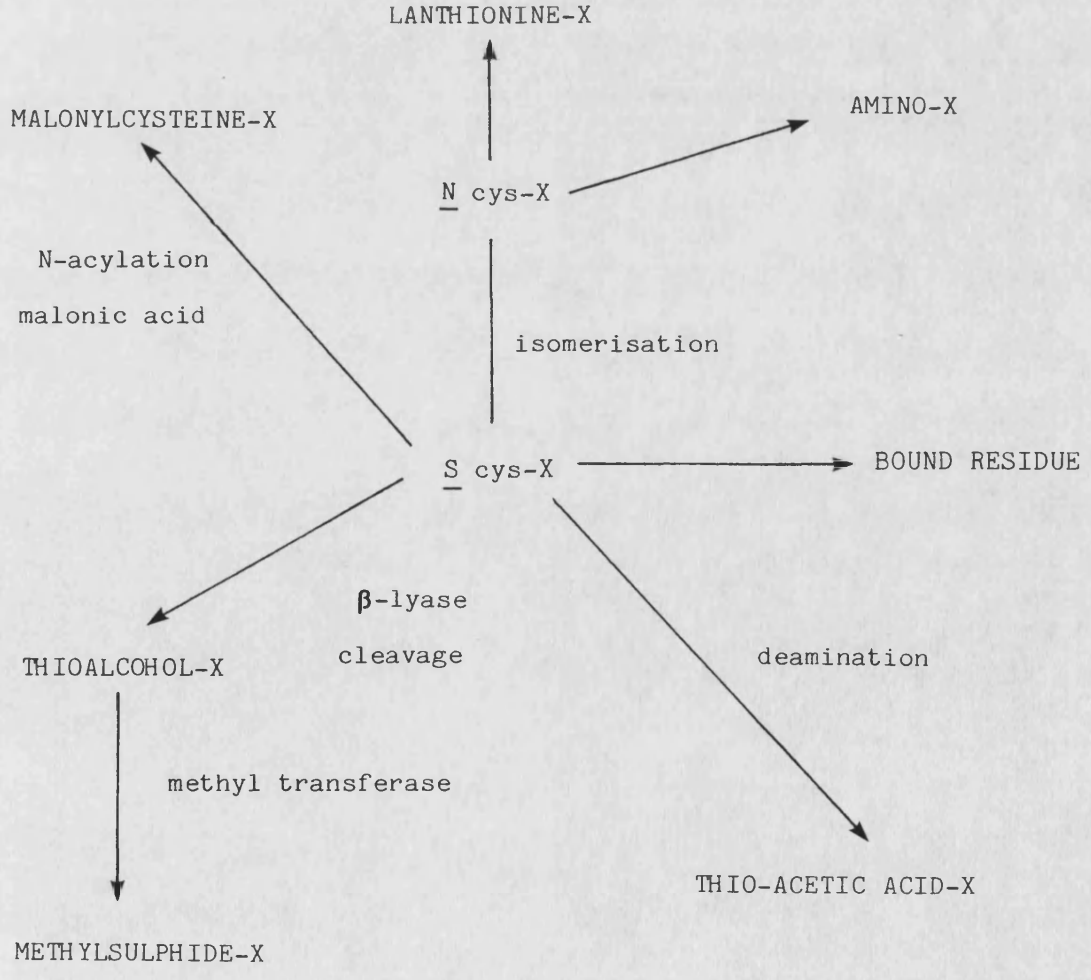
X Xenobiotic

glu glutamine

cys cysteine

gly glycine


glu
X-cys
gly
(glutathione conjugate)
maize, sorghum
(other plants?)
mammals
plants
(-glu)
(-gly)
X-cys
gly
glu
X-cys
S cys-X
(End product / further metabolism)


LANTHIONINE-X
MALONYLCYSTEINE-X
AMINO-X
N cys-X
N-acylation
malonic acid
isomerisation
S cys-X
BOUND RESIDUE
β-lyase
cleavage
deamination
THIOALCOHOL-X
methyl transferase
THIO-ACETIC ACID-X
METHYLSULPHIDE-X

enhancement of GSH and GST in maize root tissue gave a good correlation with safening activity against the thiocarbamate EPTC, however, some exceptions to the rule did occur. Stephenson, Ali and Ashton (1983) and Carringer, Rieck and Bush (1978) also reported enhanced glutathione levels in maize root tissue with dichloroacetamide treatment. Rennenberg, Birk and Schaer (1982) and Ezra and Gressel (1982) reported enhancement of GSH production in cell suspension cultures of tobacco and maize with dichlormid treatment respectively. Lay and Niland (1985) using a series of maize inbred and hybrid lines found that dichlormid enhanced GST activity in root tissue only, whereas GSH was enhanced in both root and shoot.

A number of other safener/plant combinations have been reported to elevate GSH and/or GST, particularly with dichloroacetamides, flurazole, and the N-phenylmaleamic acid prosafener N-(4-chloro-phenyl)maleimide (CPMI) e.g. Rubin, Kirino and Casida (1985), CPMI, flurazole, dichlormid X sorghum; Ezra and Stephenson (1985), dichlormid X prosomillet; Ezra et al. (1985), CDAA X maize; Gronwall et al. (1987), dichlormid, flurazole X sorghum; Dutka and Komives (1987) MG 191 X maize.

Three possible mechanisms for the enhancement of GSH levels by safeners have been suggested.

i) Enhanced glutathione synthesis:

Adams, Blee and Casida (1983) found that dichlormid, R 29148 and

other dichloroacetamides enhanced ^{35}S sulphate uptake into maize roots and metabolism to bound sulphide (cysteine and glutathione). This was related to increased activity of ATP sulphurylase, the first enzyme in the pathway for sulphate assimilation. Rennenberg, Birk and Schaer (1982) reported that dichlormid enhanced the level of enzymes involved in glutathione synthesis *in vivo* in tobacco suspension cultures. Carringer, Rieck and Bush (1978) however, found no effect of dichlormid on glutathione synthetase activity in maize root, but did find stimulation *in vitro* at levels of 5-500 nM.

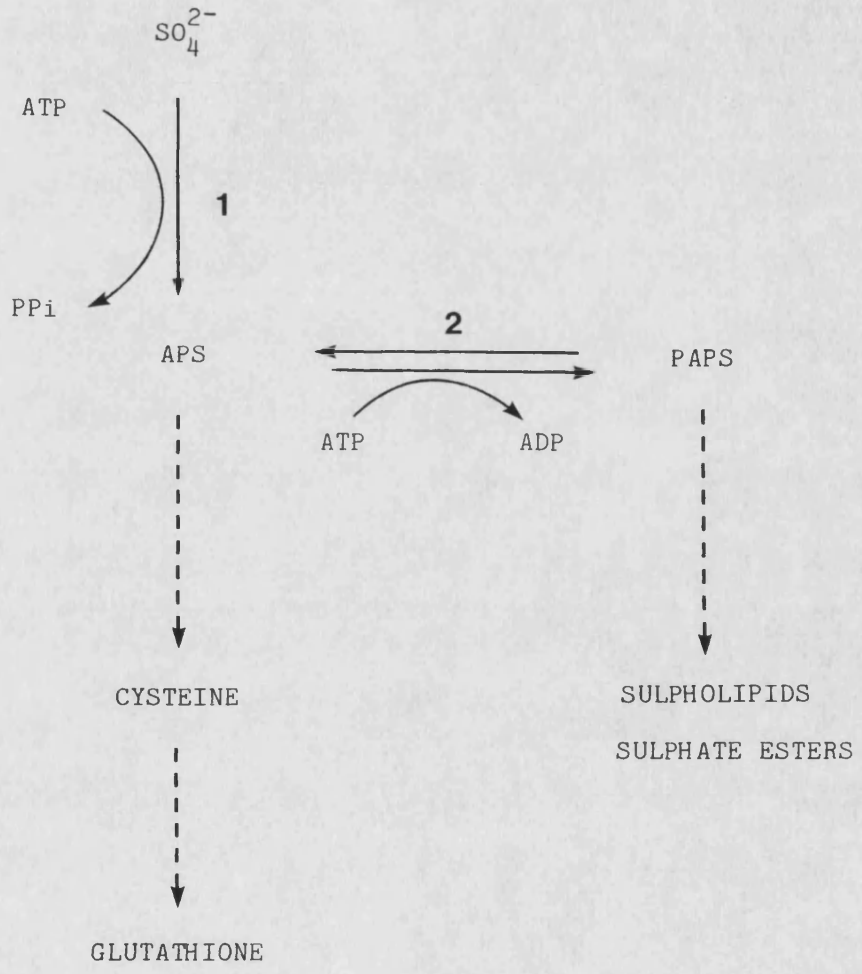
ii) Enhanced glutathione reductase activity:

As well as its role in conjugation reactions in the cell, GSH acts as a source of reducing power and is, for example, important in protecting chloroplast membranes from oxidative damage caused by H_2O_2, superoxide and other oxidants (Halliwell, 1984). GSH is oxidised to glutathione disulphide (GSSG), and reduced again to GSH by NADPH-dependent glutathione reductase (Meister and Anderson, 1983). The ratio of GSH to GSSG in the cell is related to glutathione reductase activity (Dutka and Kömives, 1987). Kömives *et al*. (1985 a, b) reported that the safener MG 191 enhanced glutathione reductase activity in maize tissue. This would make more glutathione available in its reduced form for conjugation reactions.

iii) Alteration of feedback inhibition:

Glutathione regulates its own synthesis by feedback inhibition of

Fig. 2.4 Role of ATP sulphurylase in sulphate assimilation



1. ATP sulphurylase
2. APS kinase

APS adenosine 5-phosphosulphate

PAPS 3-phosphoadenosine 5-phosphosulphate

γ-glutamyl cysteine synthetase, one of the enzymes in its metabolic pathway (Meister and Anderson, 1983). Breaux et al., (1989) suggested that flurazole, CDAA, and dichlormid, which are metabolised by GSH conjugation, may deregulate GSH feedback control, leading to enhanced GSH production. Thiocarbamate, s-triazine and chloroacetanilide herbicides have been found to elevate GSH levels in plants (Stephenson, Ali and Ashton, 1983; Stephenson and Ezra, 1985) all of which conjugate with GSH. Enhancement was to a lesser degree than with the safeners, and the herbicides did not protect against subsequent herbicide damage. It has been suggested that safeners enhance GSH levels because of their structural similarity to some herbicides, but are themselves less toxic than the herbicides (Stephenson, Ali and Ashton, 1983).

NA, cyometrinil and oxabetrinil are poor inducers of GSH, but do induce GST activity (Fedtke, 1981; Gronwald et al., 1987; Kömives et al., 1985a). Enhancement of GST activity has been reported to be associated with both enhanced activity of a constitutively present GST isoenzyme, and the induction of a novel GST isoenzyme in maize (Mozer, Tiemeier and Jaworski, 1983). Edwards and Owen (1986b, 1988) found that fenclorim treatment of maize cell cultures and dichlormid treatment of maize plants enhanced the activity of a GST isoenzyme which conjugated metolachlor, but had no effect on a GST isoenzyme which conjugated atrazine. It was suggested that the safeners were specifically regulating the expression of genes encoding for the metolachlor GST isoenzyme, but not those for the atrazine isoenzyme. Dichlormid has been found to increase the

activity of mRNA encoding GSTs (Edwards and Owen, 1988; Wiegland et al., 1986), but it was not determined if this was due to an increase in mRNA half life, or activation at a transcriptional level.

Gronwald et al. (1987) and Lay and Casida (1976) found no evidence of direct activation of GST in vitro with either dichlormid or oxabetrinil.

Because of the specificity of GST isoenzymes induced by safener treatment, the use of a general GST substrate such as chlorodinitrobenzene (CDNB) has been questioned as a measure of the ability of plants to metabolise specific herbicides (Edwards and Owen, 1986a). Gronwald et al. (1987) showed clearly that discrepancies can arise. Treatment of sorghum with flurazole, oxabetrinil, NA and dichlormid gave a doubling of GST activity towards CDNB, but values of X30, X20, X17 and X7 respectively when metolachlor was used as the substrate.

There is generally good evidence to support the hypothesis that enhancement of GSH levels and/or GST activity in safened plants contributes to the protective action of some safeners, particularly the dichloroacetamides, against the chloroactanilides and thiocarbamates. However, other evidence indicates that this might not be the only mode of action of these chemicals. Of the compounds tested by Lay and Casida (1976) several did not correspond between GSH/GST enhancement and safening ability. Some compounds which gave

good safening, produced virtually no change in GSH or GST. This included NA. Others with little or no safening activity enhanced either one or both of GSH and GST significantly. Ezra and Gressel (1982) found that although dichlormid enhanced the GSH content of maize cells in culture, this did not occur until several hours after all ^{14}C EPTC had been metabolised. They found more rapid changes in lipid biosynthesis, which counteracted EPTC effects, and this was suggested as the primary mode of dichlormid action. The protective action of safeners against herbicides which are not metabolised via the GSH/GST system, e.g. chlorsulfuron (Hatzios, 1983c) would also be difficult to explain in terms of changes in this system alone.

b) Mixed Function Oxidases

The cytochrome P450 mixed function oxidases (MFOs), which have been found to be involved in the metabolism of xenobiotics in mammalian systems, are also believed to be involved in the metabolism of many herbicides in plants (Hatzios and Penner, 1982). They mediate in a number of types of reaction; hydroxylation, oxidation, deamination, dealkylation and participate in important biosynthetic pathways which include gibberellin and unsaturated fatty acid biosynthesis (Hatzios and Penner, 1982; Hendry, 1986; Kömives and Dutka, 1989).

The involvement of MFOs in safener action has been suggested by a number of workers, though the evidence at present is still unclear (Kömives and Dutka. 1989). Sweetser (1985) reported that NA,

dichlormid and cyometrinil reduced significantly the half-life of chlorsulfuron and related sulfonylurea herbicides in treated maize and wheat. Metabolism in both species had previously been found to involve an initial hydroxylation followed by glucose conjugation (Sweetser, Schow and Hutchinson, 1982), and it was suggested that an MFO system was involved. Sweetser (1985) concluded on this evidence that safener protection against these herbicides involved an induction of MFO activity. However, other workers have disputed these results. Frear, Swanson and Mansager (1987) found no enhancement of chlorsulfuron metabolism in maize associated with NA treatment, and Kömives et al. (1985a) reported no change in MFO levels in maize with NA and dichlormid.

The metabolism of chlortoluron in wheat which involves MFO activity (Cole, 1983; Cabanne et al., 1985) was both enhanced and inhibited in suspension cultures with the addition of 2,4-D, cyometrinil or dichloromid, depending upon the time of application (Canivenc et al., 1988).

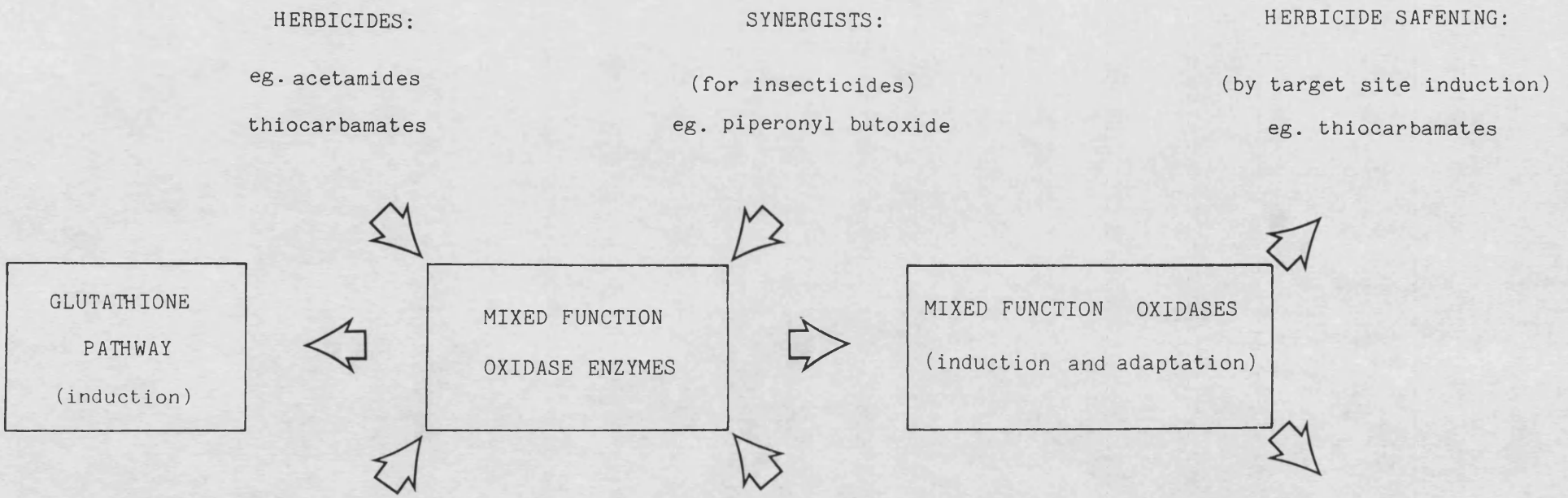
Safener protection against EPTC has been suggested to involve an enhancement of MFO mediated sulfoxidation of the parent herbicide, rather than any changes in GST activity or GSH levels (Leavitt and Penner, 1979; Dutka and Kömives, 1983). Conjugation of the sulfoxide with GSH in vitro was rapid suggesting that its detoxification in maize could occur without prior safener treatment to enhance GSH levels. EPTC was more toxic to maize seedlings than its sulfoxide or sulfone. Hatzios (1983a) reported that ozone and

the MFO inhibitors piperonyl butoxide (PBO) and propyl gallate antagonised the safening effect of dichlormid against EPTC in maize.

This was thought to be due to the compounds counteracting the effect of dichlormid on the sulfoxidation of EPTC. However, similar results were found for the interaction of these MFO inhibitors with cyometrinil and metolachlor in sorghum (Hatzios, 1983b), even though there is no evidence for oxidative activation being involved in the metabolism of chloroacetanilides (Gronwald, 1989). The inhibitory effect of ozone on MFOs and the specificity of the other MFO inhibitors against plant sulfoxidases has been questioned, as well as the assumption that MFOs are involved in EPTC sulfoxidation (Gronwald, 1989).

Fedtke and Trebst (1987) and Fedtke (1987) have proposed a model to explain herbicide and safener action based around the mixed function oxidases (Fig. 2.5). They found that MFO inhibitors acted as safeners against some herbicides in an oat rooting test system. Conversely some safeners have been reported to act as insecticide synergists, suggesting that they may act as MFO inhibitors (Ketchersid, Plapp and Merkle, 1985). Fedtke and Trebst (1987) suggested that herbicides, safeners, and synergists all acted as MFO inhibitors. However, with safeners, an initial inhibition of MFOs was followed by MFO induction which produced the safening agent. Although this idea of MFO induction following MFO inhibition has been reported to occur in insects and animals (Fedtke, 1987)

Fig. 2.5 Proposed role of mixed function oxidases in herbicide safening and synergism (adapted from Fedtke and Trebst, 1987)


HERBICIDES:
eg. acetamides
thiocarbamates
SYNERGISTS:
(for insecticides)
eg. piperonyl butoxide
HERBICIDE SAFENING:
(by target site induction)
eg. thiocarbamates
GLUTATHIONE
PATHWAY
(induction)
MIXED FUNCTION
OXIDASE ENZYMES
MIXED FUNCTION OXIDASES
(induction and adaptation)
SAFENERS:
dichlormid
NA etc.
SYNERGISTS:
(for herbicides)
eg. 1-aminobenzotriazole
HERBICIDE SAFENING:
(by increased degradation)
eg. sulfonylureas

this model requires further evidence to substantiate in plant systems, particularly with respect to some of the data produced with the oat rooting test system.

2.3.4 Physiological and Competitive Antagonism of Herbicides

Several sites for the antagonism of herbicide action by herbicide safeners have been investigated. This is in part due to the apparently diverse mode of action of the thiocarbamate and chloroacetanilide herbicides. Their effects are consistent with a chronic loss of vital metabolic processes, rather than inhibition of a central metabolic reaction (Wilkinson, 1978). Lipid, terpenoid, protein and nucleic acid synthesis, as well as membrane function and ion transport are amongst the physiological and biochemical processes which have been found to be affected by these herbicides (Hatzios, 1989b).

Many of the effects of thiocarbamates upon plant biochemical processes resemble modified gibberellin (GA) availability (Wilkinson, 1983). Wilkinson and Ashley (1979) found that EPTC modified GA biosynthesis in wheat. Mevalonic acid (MVA) incorporation into kaurenoids was reduced, and there was a corresponding accumulation of kaurene. This was found to be due to EPTC inhibition of kaurene oxidase activity. Metolachlor inhibition of sorghum growth could also be partially explained by an inhibition of GA biosynthesis (Wilkinson, 1981). Cyometrinil reversed the inhibition of GA biosynthesis in sorghum by

metolachlor and alachlor (Wilkinson, 1981, 1982). Dichlormid alone was found to have no effect upon ^{14}C MVA incorporation into kaurene in a cell free GA precursor biosynthetic system from sorghum, but totally reversed the inhibitory effect of EPTC upon this system when applied in combination (Wilkinson, 1983). Further evidence for the effect of thiocarbamates upon GA biosynthesis and reversal by safeners was found in the reversal of EPTC induced growth inhibitions in sorghum by exogenous application of GA (Wilkinson, 1989).

Lipid biosynthesis has been reported to be another site of interaction between herbicide safeners and the thiocarbamates and chloroacetanilides. Ezra and Gressel (1982) found that dichlormid had a more rapid effect on maize cell cultures in counteracting EPTC induced inhibition of lipid metabolism, than its effect upon glutathione synthesis. Further investigation revealed that dichlormid partially counteracted EPTC inhibition of ^{14}C acetate incorporation into neutral lipids, but not its effect upon polar lipids in this system (Ezra, Gressel and Flowers, 1983). However, this was not thought to account for the complete mode of action of the safener. Conversely, Warmund, Kerr and Peters (1985) reported no effect of alachlor or flurazole on the total lipid or fatty acid composition of sorghum. However, alachlor induced a greater triglyceride to phospholipid ratio, and this was partially overcome by flurazole. Zama and Hatzios (1986b) using isolated soybean leaf mesophyll cells found enhanced lipid metabolism with cyometrinil, oxabetrinil and flurazole treatments. All safeners inhibited this

process at the highest concentration, but as inhibition was not distinct, it was not thought to be the primary site of action. Oxabetrinil partially reversed metolachlor inhibition of lipid biosynthesis in sorghum protoplasts (Zama and Hatzios, 1987) and EPTC inhibition of ^{14}C acetate incorporation into the fatty acids of isolated spinach chloroplasts was counteracted by NA, and dichlormid (Wilkinson and Smith, 1975).

Other researchers have emphasised the importance of epicuticular wax formation as a site of safener/herbicide interaction. Dichlormid prevented EPTC induced aggregation of epiculticular waxes of maize (Leavitt and Penner, 1978). Barta, Kőmives and Dutka (1983) found reduced ^{14}C incorporation into the C_{32} component of the ester and aldehyde fraction of maize epicuticular waxes with EPTC application. Both dichlormid and MG 191 reversed the EPTC induced effect. Ebert and Ramsteiner (1984) reported similar findings for the reversal of metolachlor effects upon sorghum leaf epicuticular waxes by cyometrinil. Metolachlor inhibited formation of C_{28} and longer chain alcohols, and C_{30} and C_{32} fatty acids. Simultaneous treatment with cyometrinil resulted in normal wax synthesis.

Changes in other metabolic processes associated with safener treatment have been reported (Zama and Hatzios, 1986b). Cyometrinil, oxabetrinil and flurazole enhanced CO_2 fixation, RNA, DNA and protein biosynthesis in isolated soybean leaf cells. Metolachlor inhibited DNA and RNA synthesis in isolated sorghum

protoplasts, and these effects were partially counteracted by oxabetrinil (Zama and Hatzios, 1987).

Inhibition of acetohydroxyacid synthetase (AHAS) that is involved in the biosynthesis of the branched chain amino acids, valine, leucine and isoleucine, has been identified as the mode of action of imidazolinone and sulfonylurea herbicides (Ray, 1982; Shaner, Anderson and Stidham, 1984). Rubin and Casida (1985) and Frear, Swanson and Mansager (1987) have investigated AHAS as a possible site of safener antagonism of these herbicides. Neither NA nor dichlormid in vitro altered chlorsulfuron inhibition of AHAS. Maize pretreated with dichlormid contained enhanced levels of AHAS (25%) but the enzyme retained its chlorsulfuron sensitivity, so there was no evidence for the induction of sulfonylurea resistant AHAS isoenzymes with safener treatment (Rubin and Casida, 1985). The enhanced level of AHAS in safened maize was not thought to be sufficient to provide the level of safener protection observed.

The antagonism of auxin-like herbicices and herbicides such as the thiocarbamates and diclofop-methyl (Beste and Schreiber, 1970; Taylor and Loader, 1984) may be due to a physiological antagonism of RNA synthesis. Thiocarbamates inhibit RNA synthesis, whilst 2,4-D and other auxin-like herbicides stimulate RNA synthesis (Beste and Schreiber, 1972). The possibility that safeners may be acting in a "hormonal-like" way has been suggested by Frear, Swanson and Mansager (1987). NA was found to induce an auxin-like gravitropic response in maize roots, and the auxins indoleacetic

acid (IAA), phenylacetic acid (PAA), indolebutyric acid, and β-naphthalene-acetic acid provided partial protection against chlorsulfuron in maize. Other reports of plant hormones antagonising herbicide activity have been made (Field and Caseley, 1987; Donald and Fawcett, 1975; Wilkinson, 1978).

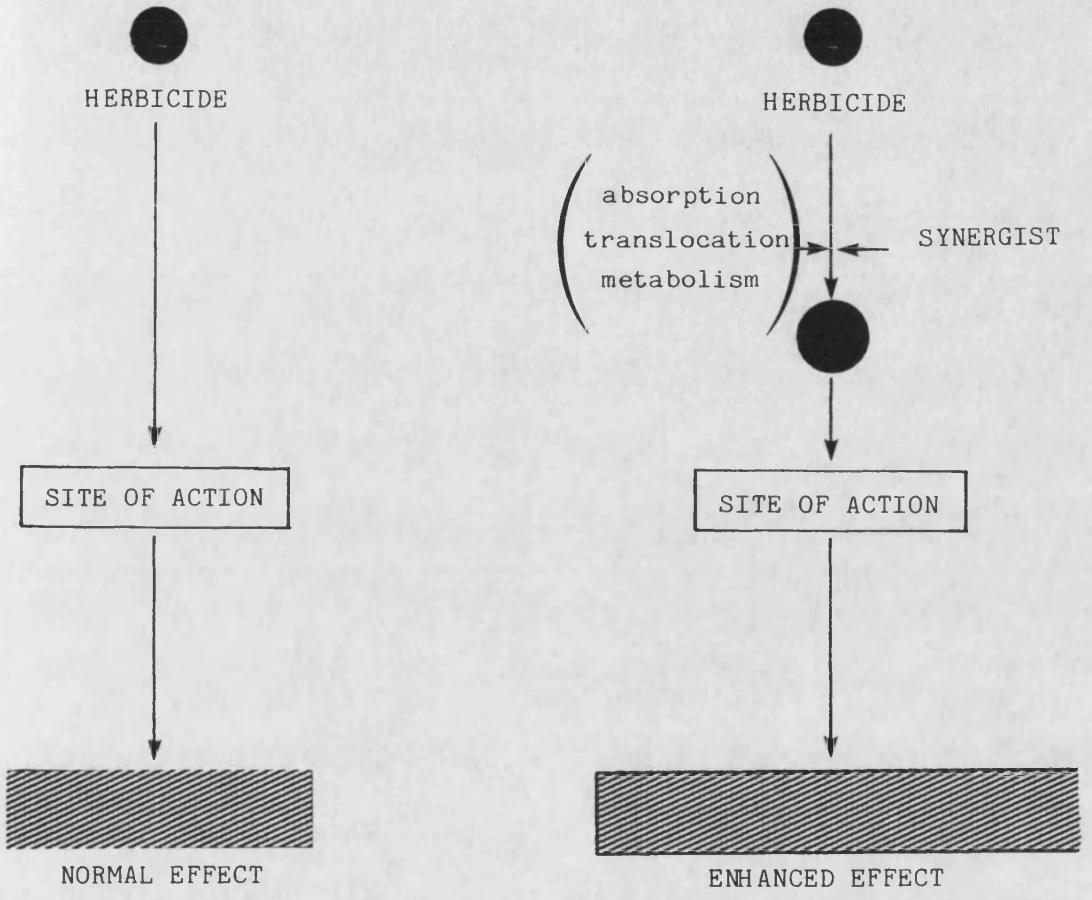
2.4 MECHANISMS OF ACTION OF HERBICIDE SYNERGISTS

2.4.1 An Overview

The mechanisms by which synergists enhance the phytotoxicity of herbicides may be analogous but opposite to those described in Section 2.3.1 for herbicice safeners. Enhancement of herbicide uptake and/or translocation to sites of action, and reduction in the rate of herbicide detoxification were the most likely mechanisms for a range of herbicide/agrochemical synergistic interactions reviewed by Hatzios and Penner (1985) (Figure 2.6). Synergism arising from interactions occurring at the physiological or biochemical site of action of herbicides may also occur, but are less well documented.

The following section outlines the mechanisms which are believed to be involved in the synergistic action of some of the herbicide/herbicide and herbicide/adjuvant combinations described in Section 2.2.2.

Fig. 2.6 Proposed mechanisms of herbicide synergist action (after Hatzios and Penner, 1985)



(Synergist enhances absorption, translocation or inhibits metabolism of the herbicide)

2.4.2 Specific Examples

Donnaley and Ries (1964) reported the synergism of amitrole activity in Agropyron repens and other perennial weeds by ammonium thiocyanate. Enhanced herbicide activity was associated with increased translocation of amitrole. Babiker and Duncan (1975) and Cook, Stephen and Duncan (1981) also attributed the synergism of amitrole in bracken (Pteridium aquilinum) by ammonium thiocyanate to enhanced uptake and translocation of the herbicide. Carter (1975) has suggested that the increased mobility of amitrole may be a secondary effect, due to inhibition of degradation of the parent herbicide to its less mobile and toxic metabolite 3-aminotriazole-alanine. However, changes in uptake and translocation of amitrole may not be the only mechanisms involved in ammonium thiocyanate synergism. Forde (1966) has disputed the findings of Donnaley and Ries (1964) and reported a decrease in amitrole translocation in A. repens. Cook and Duncan (1979) have proposed that synergism is due to preferential oxidation of ammonium thiocyanate within the plant, thus preventing formation of amitrole free radicals and subsequent conjugation of amitrole with serine to form 3-aminotriazole-alanine.

The synergism of propanil activity in rice (Oryza sativa) by organophosphate and carbamate insecticides has been reported to be due to inhibition of herbicide metabolism. Hydrolysis of propanil was found to be inhibited in rice by these insecticides (Matsunaka, 1969), and Frear and Still (1968) attributed this to direct

inhibition of an aryl acylamidase. Hatzios and Penner (1985) cited several other examples of synergism of herbicides by organophosphate and carbamate insecticides which were associated with inhibition of herbicide metabolism. These included monuron, linuron, alachlor, butylate, dicamba, metribuzin and pyrazon. The synergistic activity of the herbicide tridiphane has also been found to be due to inhibition of herbicide detoxification. Tridiphane synergises the activity of atrazine, EPTC and alachlor in a range of problem grass weeds - giant foxtail (Setaria faberi), large crabgrass (Digitaria sanguinalis) and proso millet (Panicum miliaceum) (Dekker, 1984; Ezra, Dekker and Stephenson, 1985; Zoner, 1983). Changes in herbicide uptake do not appear to account for the synergistic activity of tridiphane. Boydston and Slife,(1986) found increased atrazine uptake in giant foxtail with tridiphane pretreatment. However, Zorner (1983) reported reduced atrazine uptake with tridiphane pretreatment and McCall et al. (1986) found no change in atrazine uptake with simultaneous application of tridiphane.

All of the synergised herbicides are metabolised via glutathione conjugation (Hatzios and Penner, 1982). Zorner (1983) found that tridiphane inhibited in vivo detoxification of atrazine in giant foxtail, and also the in vitro activity of glutathione-s-transferase (GST) towards the herbicide. Boydston and Slife (1986) and McCall et al. (1986) reported similar inhibition of atrazine metabolism in giant foxtail by tridiphane, but no effect was observed upon atrazine metabolism in maize (Boydston and Slife,

1986). GST isolated from maize leaves had X14 the specific activity towards atrazine than GST from giant foxtail, but both enzymes *in vitro* had a similar I_{50} with tridiphane, i.e. similar sensitivity to tridiphane (Boydston and Slife 1986). Lamoureux and Rusness (1986b) found that tridiphane was metabolised by glutathione (GSH) conjugation, and that both tridiphane and its GSH conjugate inhibited GST activity towards atrazine *in vitro*. As a competitive inhibitor of GST with respect to GSH, the GSH-tridiphane conjugate was four times as effective on giant foxtail GST as on maize GST. The GSH-tridiphane conjugate was also found to be a potent inhibitor of GSTs from other sources (Lamoureux and Rusness, 1986b) and of GST mediated metabolism of the insecticide diazinon in the house fly (*Musca domestica*) (Lamoureux and Rusness, 1987).

The selectivity of tridiphane activity between maize and grass weeds has been attributed to a combination of factors (Lamoureux and Rusness, 1986b). Tridiphane and its GSH conjugate inhibit GST from crop and weed plants, and both types of plant metabolise tridiphane via GSH conjugation. However, the GSH-tridiphane conjugate was more stable in giant foxtail extracts than those from maize, suggesting that catabolism of the tridiphane conjugate was more rapid in maize (Lamoureux and Rusness, 1986b). This, together with higher GSH levels in maize leaves, would allow more rapid metabolism of both tridiphane and atrazine in maize. The greater specific activity of maize GST to atrazine (Boydston and Slife, 1986), and alternative pathways for atrazine metabolism in maize (Hatzios and Penner, 1982) may also contribute to the selective

nature of tridiphane synergism.

MFOs are involved in oxidation reactions which can either activate or deactivate xenobiotics in insects and mammals. They are also believed to be involved in similar mechanisms in plants (Hatzios and Penner, 1982), although much of this evidence is circumstantial (Cole, Edwards and Owen, 1987). MFO inhibitors have been used to synergise the activity of herbicides in plants, and in particular, the phenylureas chlortoluron and isoproturon. Gaillardon et al. (1985) synergised the activity of chlorotoluron in wheat using piperonyl butoxide (PBO) - an insecticide synergist - and aminobenzotriazole (ABT) - an enzyme activated MFO inhibitor. ABT also synergised isoproturon toxicity in wheat. Cabanne et al. (1987) reported that ABT inhibited the metabolism of isoproturon and chlortoluron in wheat by inhibiting the enzymes for ring alkyl hydroxylation and the second N-demethylation of isoproturon. ABT, 3-(2,4-dichlorphenoxy)-1-propyne (2,4-DP) and tetcylasis all inhibited the metabolic breakdown of chlortoluron in maize and cotton cell suspension cultures (Cole and Owen, 1987b). Kemp and Caseley (1987) demonstrated that ABT could be used to overcome the tolerance of a resistant population of blackgrass (Alopecurus myosuroides) to isoproturon and chlortoluron. Other MFO inhibitors also proved to be effective (Kemp, Newton and Caseley, 1988).

The use of MFO inhibitors to synergise herbicide activity is an example where targeting a particular metabolic process has been used to alter herbicide activity. This compares to other herbicide/

synergist/safener combinations, where the mechanisms involved have been elucidated after an interaction was observed.

Synergism between glyphosate and imidazolinone herbicides (Bocion, 1987) may be an example of physiological synergism. Both herbicides inhibit the biosynthesis of different amino acids. The imidazolinones inhibit acetohydroxyacid synthetase activity and production of the branched chain amino acids valine, leucine and isoleucine (Shaner et al., 1985), whereas glyphosate inhibits biosynthesis of aromatic amino acids via the shikimic acid pathway (Dodge, 1983b). Gressel and Shaaltiel (1988) suggested that synergism might be due either to a superior starvation of amino acids, or a synergistic build up of toxic intermediates resulting from the double blockage.

2.5 AIMS AND OBJECTIVES OF THIS STUDY

Despite all the research which has been carried out into the mode of action of herbicide safeners, it is still not certain how these chemicals modify the activity of herbicides in plants. There is much evidence to suggest that safeners enhance the metabolism of herbicides, in particular those metabolised via glutathione conjugation. However, other responses, including physiological antagonism of herbicide effects, and alteration of herbicide target site proteins have also been suggested. No one simple mechanism appears to explain all safener x herbicide x plant combinations, and it is probable that safeners induce a range of effects in treated plants, some or all of which contribute to enhanced

herbicide tolerance.

The aim of this investigation was to look at some of the physiological and biochemical responses induced in plants by safener and other chemical treatments, and assess their possible effects on herbicide activity.

The work comprised of three main areas of research.

1. Whole plant safener responses

Chemical and botanical specificity of safener action was investigated using examples of different safener compounds against a range of herbicides, on Zea mays and a grass species, Echinochloa crus-galli. As well as looking for protective action against herbicide toxicity, it was hoped that safener effects upon seed germination and plant growth might provide clues to the mechanisms involved in plant responses to safeners.

2. Biochemical effects

The effects of naphthalic anhydride (NA) and dichlormid on glutathione levels and glutathione-s-transferase activity in maize were investigated as a mechanism for protection against herbicides metabolised via glutathione conjugation. Other chemicals were evaluated as potential modulators of glutathione in plants, and their potential as herbicide synergists and antagonists assessed.

The effects of NA and dichlormid on acetohydroxyacid synthetase (AHAS) were also investigated as a possible site of safener antagonism of the sulfonylurea herbicides.

3. Effect of NA on plant cell growth

The physiological effect of NA on cell growth was assessed using a suspension cell culture of maize. It was hoped that the growth effects of NA and interaction with herbicides in the culture system might provide further evidence for mechanisms involved in NA action. This also provided an opportunity to assess this type of tissue culture system as a potential method of screening for herbicide x safener interactions.

3. THE EFFECTS OF SELECTED HERBICIDE SAFENER AND HERBICIDE COMBINATIONS ON THE GERMINATION AND GROWTH OF ECHINOCHLOA CRUS-GALLI and ZEA MAYS

3.1 INTRODUCTION

Herbicide safeners exhibit a high degree of chemical and botanical specificity in their range of protective action (Hatzios, 1983c). Generally, safening is restricted to the "growth inhibitor-type" herbicides such as the thiocarbamates and chloroacetanilides, and it is almost exclusively limited to monocot species, in particular the large grained cereals, maize, sorghum, rice, oats, wheat and barley.

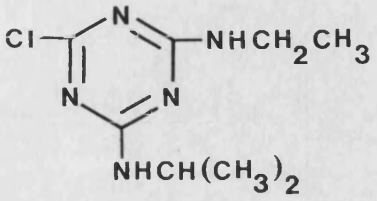
Chemical and botanical specificity varies between the individual safener compounds. The oxime ethers, cyometrinil and oxabetrinil and the 2,4-disubstituted 5-thiazolecarboxylate, flurazole, give good protection to grain sorghum against chloroacetanilide herbicide injury, but poor protection to maize and other cereals, and to sorghum against other herbicides. Conversely, the dichloroacetamide dichlormid gives good protection to maize against thiocarbamates and chloroacetanilides, but almost no protection to sorghum against any herbicides. Naphthalic anhydride (NA) exhibits the broadest range of species and herbicides in its protective action, safening maize, sorghum, rice, oats and wheat against some or all of these herbicides (Hatzios, 1983c).

Echinochloa crus-galli is a small seeded (3 mg individual seed weight) weed grass. The oxime ethers and flurazole have been reported to protect some grass weeds including alexander grass (Bruchiaria plantaginea), Eleusine spp., prosomillet (Panicum miliaceum L.) and Sorghum spp. against acetanilide injury (Nyffeler, Gerber and Hensley, 1980; Dill et al., 1982; Schafer, Brinker and Radke, 1980). Parker (1983) also reported success in safening the small grained cereal tef (Eragrostis tef (Zucc.) Trotter), with an individual seed weight of 0.4 mg, with NA. However, there are few reports of the use of Echinochloa crus-galli in safener trials.

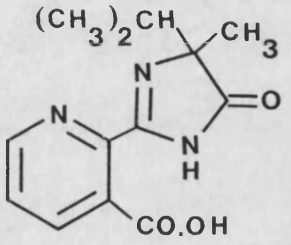
The aim of this initial section of work was to assess the level of protection provided by a number of different safeners to maize and Echinochloa crus-galli against examples of selected herbicide groups. Three safeners were used; NA, which has a broad spectrum of action, and oxabetrinil and flurazole, which are predominantly safeners for sorghum. These were assessed for safening against four herbicides; chlorsulfuron (sulfonylurea), imazapyr (imidazolinone), metolachlor (acetanilide) and atrazine (s-triazine) (Fig. 3.1), which represented three "growth inhibitor-type" herbicides, and one "photosynthetic inhibitor-type". A separate trial of the safener dichlormid on maize with chlorsulfuron was also conducted.

Some phytotoxic effects of safeners on treated plants have been reported (Hatzios, 1983c). NA applications in maize have been found to cause a slight reduction in plant growth (Hickey and Krueger,

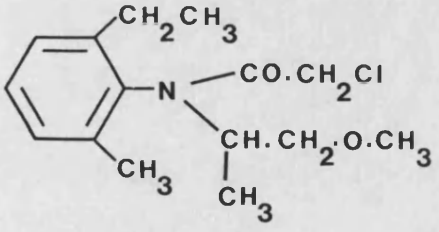
Fig. 3.1 Herbicides used in the safener assessment



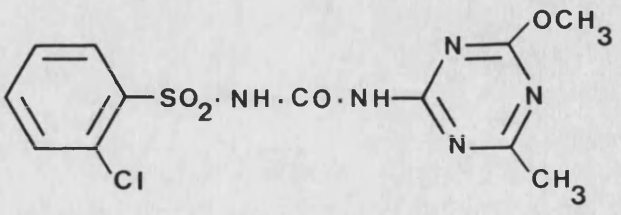
ATRAZINE



IMAZAPYR



METOLACHLOR



CHLORSULFURON

1974). The oxime ether cyometrinil at high rates of application caused reduction in seed viability of Sorghum spp. (Dill et al., 1982; Davidson et al., 1978). Oxabetrinil was reported to have no adverse effect on sorghum seed viability (Dill et al., 1982), and neither did flurazole on sorghum germination or growth (Schafer, Brinker and Radke, 1980, 1981). However, both these safeners had measurable effects on growth and respiration of sorghum seedlings during imbibition and early germination (Ketchersid and Merkle, 1983).

In view of this, care was taken to record treatment effects upon seed germination and plant growth, to assess if any of the safeners caused a reduction in germination rates and early plant growth. Measurements of leaf extension during the trial period, as well as plant fresh and dry weights at the end, were also used for this purpose.

3.2 MATERIALS AND METHODS

3.2.1 Plant Material

Maize (Zea mays L.) var. LGII was used in both growth trials one and two. Echinochloa crus-galli used in the first growth trial was obtained from the Tropical Weeds Group at Long Ashton, and was from seed collected from plants grown in the Weed Research Organisation glasshouses at Yarnton in 1976.

3.2.2 Soil and Growth Conditions

(i) Experiment 1. (Long Ashton)

The first growth trial was carried out during August and September 1986 in a glasshouse at Long Ashton Research Station, Bristol.

Seeds were sown in 8 cm pots containing a Mendip silt loam with 15% v/v sand. Fertilizer, Vitax Q4, was added at a rate of 3.3 g per litre. Two maize seeds were sown per pot at a depth of three centimetres. Echinochloa crus-galli seeds were "raked in" to the soil surface at a rate of 5 seeds per pot.

(ii) Experiment 2. (Bath)

The second growth trial was carried out during July and August 1987 in a glasshouse at Bath University. Two maize seeds were planted at 3 cm depth in 8 cm pots. Soil used contained five parts of a loam collected from a Bathampton field site, to one part sand. A slow release fertilizer with NPK of 14, 14, 14 was added at a rate of 4 kg/m^3.

In both experiments, plants were watered daily as required.

3.2.3 Chemicals and Application Rates

(i) Safeners.

Chemical safeners and formulations used are summarised in Table 3.1.

Before applying safeners as a seed dressing, maize seeds were washed in running tap water for 15 minutes and dried quickly to remove a fungicide seeddressing of captan and thiram. This treatment had no subsequent effect upon seed germination.

NA, oxabetrinil and dichlormid were applied to maize seeds at rates of 0, 0.25, 0.5 and 1.0% by seed weight. Flurazole was applied at rates of 0, 0.5, 1.0 and 2.0% by seed weight. Echinochloa crus-galli seeds were dressed at rates of 0, 0.25, 0.5 and 1.0% for NA and 0, 0.5, 1.0 and 2.0% for oxabetrinil and flurazole.

Safeners were applied by shaking a weighed sample of seeds in a small glass flask with a corresponding amount of safener. Seeds were then removed from the flask and reweighed, and more safener applied until the correct amount of chemical was adhering to the seed surface. Seeds were stored in their shaking receptacles until planting.

Table 3.1 Chemical safeners and formulations used

Safener	Formulation		Company
NA	Powder	97% w/w ai	Gulf Oil Co.
Flurazole (MON 4606)	Liquid	480 g/litre	Monsanto
Oxabetrinil (CGA 92194)	Powder	50% w/w ai	Ciba-Geigy
Dichlormid (R 25788)	Powder	20% w/w ai	Stauffer

Table 3.2 Herbicides and formulations used

Herbicide	Formulation	Company
Chlorsulfuron	20% W.P.	Du Pont
Metolachlor	500 g/litre E.C.	Ciba-Geigy
Atrazine	500 g/litre E.C.	Ciba-Geigy
Imazapyr	250 g/litre E.C.	American Cyanamid

(ii) Herbicides

Herbicides and formulations used are summarised in Table 3.2. All herbicides were applied pre-emergence two days after sowing; chlorsulfuron and imazapyr at rates of 0, 0.01, 0.04 and 0.16 kg/ha, atrazine and metolachlor at 0, 0.25, 1.0 and 4.0 kg/ha.

The Long Ashton track sprayer was used in Experiment 1 fitted with a Teejet 800 2E nozzel and spraying at 373 litres/ha. Chlorsulfuron was applied in Experiment 2 using a track sprayer at Bath University fitted with a Teejet 800 15 LP nozzel, and spraying at 610 litres/ha.

3.2.4 Experimental Design

Experiment 1 was a multifactorial trial of four herbicides (metolachlor, atrazine, chlorsulfuron and imazapyr) applied at four levels, with three safeners (NA, oxabetrinil and flurazole) applied at four levels, on both maize and Echinochloa crus-galli. Two replicates were used per treatment. Pots for each species were laid out in a randomised block design with the replicates as blocks.

Experiment 2 was a multifactorial trial of chlorsulfuron applied at four levels with dichlormid applied at four levels. Five replicates were used per treatment, and pots were laid out in a randomised block design with replicates as blocks.

3.2.5 Data Collection

(i) Germination

The number of seeds germinated per pot was recorded on days 9 and 13 after sowing for maize and Echinochloa crus-galli respectively in Experiment 1, and 10 days after sowing for maize in Experiment 2.

(ii) Extension of third leaf

Figure 3.2 depicts how measurement of the growth of the third leaf to emerge on maize plants was recorded. Values were taken as the height of the tip of the third leaf above the soil surface when it was held up vertically against a ruler.

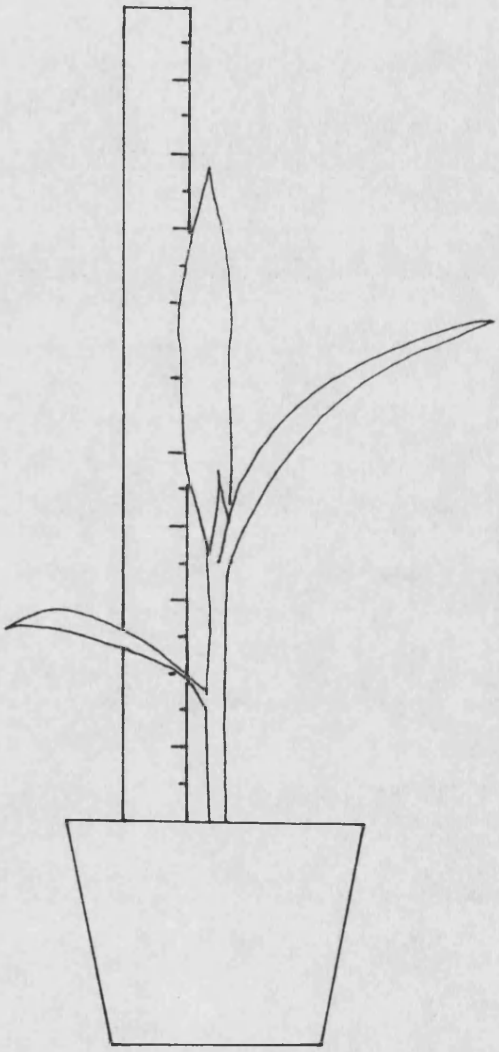
Measurements were taken on days 14, 18 and 24 after sowing in Experiment 1, and days 14, 16, 18, 21 and 23 in Experiment 2.

(iii) Fresh and dry weights

Maize plant shoots were harvested on days 34 and 25 after sowing in Experiments 1 and 2 respectively. Echinochloa crus-galli plants were harvested 54 days after sowing.

Shoot fresh weights per pot were recorded at the time of harvest, and dry weights after 48 hours drying at 105°C.

Fig. 3.2 Method of measuring maize third leaf length



3.2.6 Analysis of Data

All data collected was subjected to analysis of variance (anova) using a Genstat 5 programme on the VAX/VMS System at Long Ashton.

3.3 RESULTS

3.3.1 Experiment 1. Echinochloa crus-galli

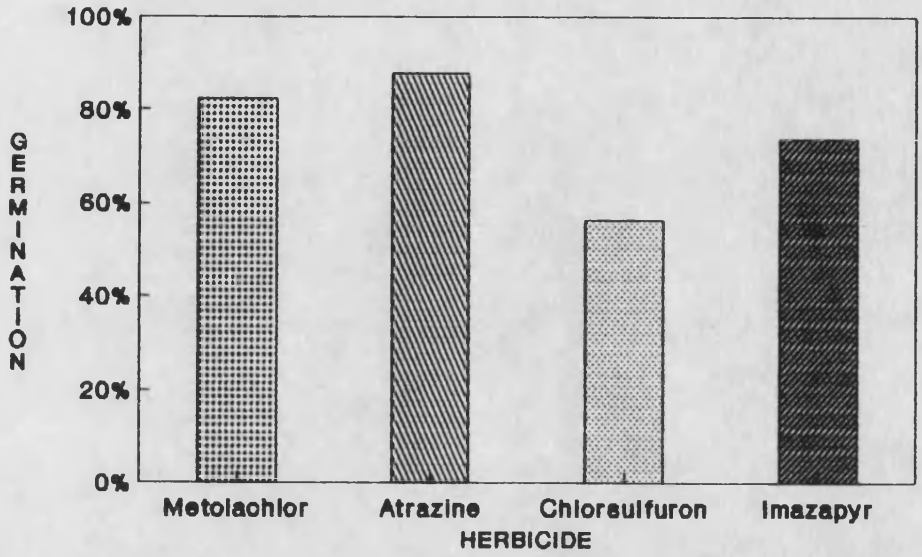
3.3.1.1 Effect of herbicides and safeners upon germination

Anova revealed a highly significant effect ($P < 0.001$) attributable to herbicides upon Echinochloa crus-galli germination. Both imazapyr and chlorsulfuron treatments significantly reduced germination compared to atrazine and metolachlor treatments (Fig. 3.3).

Comparison of safener treatments showed a significant reduction in germination with flurazole as compared to NA and oxabetrinil (Fig. 3.4).

Although germination rates for individual herbicide safener combinations showed particularly low values for chlorsulfuron x oxabetrinil/flurazole and imazapyr x flurazole, anova indicated no significant herbicide x safener interaction effect (Table 3.3).

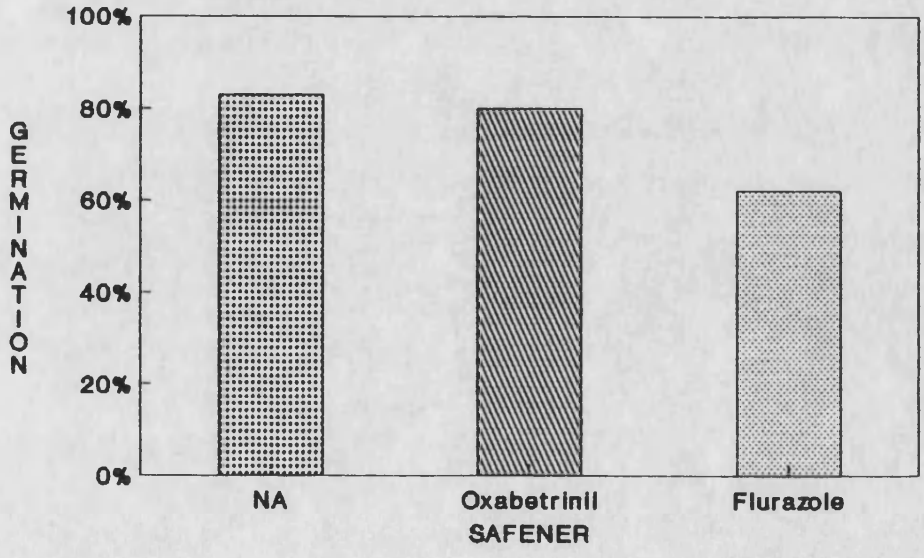
Fig. 3.3 Effect of herbicides on
Echinochloa crus-galli germination
13 days after sowing



anova: Fpr. < 0.001

control germination = 85%

Fig. 3.4 Effect of safeners on
Echinochloa crus-galli germination
13 days after sowing



anova: Fpr. < 0.001

control germination = 85%

Table 3.3 Germination of Echinochloa crus-galli with herbicide x safener treatments

Safener / Herbicide	% Germination[1] NA	Ox	Fl
Met.	88.8	87.5	71.3
Atr.	93.1	94.4	76.3
Chl.	70.6	60.0	38.7
Ima.	80.0	78.8	62.5

anova (herbicide x safener)

F.pr. = 0.316

(1) Average of 16 values.

Control germination = 85%

Table 3.4 Effect of safener level on Echinochloa crus-galli germination for each herbicide treatment

Herbicide	Safener	% Germination[2] Safener level[1] 0	1	2	3
Met.	NA	87.5	95.0	92.5	80.0
	Ox	90.0	87.5	85.0	87.5
	Fl.	82.5	80.0	65.0	57.5
Atr.	NA	97.5	92.5	87.5	95.0
	Ox	82.5	97.5	100	97.5
	Fl.	92.5	57.5	90.0	65.0
Chl.	NA	57.5	75.0	75.0	75.0
	Ox	47.5	70.0	70.0	52.5
	Fl.	45.0	42.5	35.0	32.5
Ima.	NA	72.5	85.0	82.5	80.0
	Ox	70.0	87.5	77.5	80.0
	Fl.	70.0	47.5	72.5	60.0

anova: Fpr. = 0.007

(1) Safener levels	0	1	2	3
Ox and Fl.	0	0.5	1.0	2.0% seed wt.
NA	0	0.25	0.5	1.0% seed wt.

(2) Average of 4 values.

Fig. 3.5 Effect of chlorsulfuron level on Echinochloa crus-galli germination at 13 days with each safener

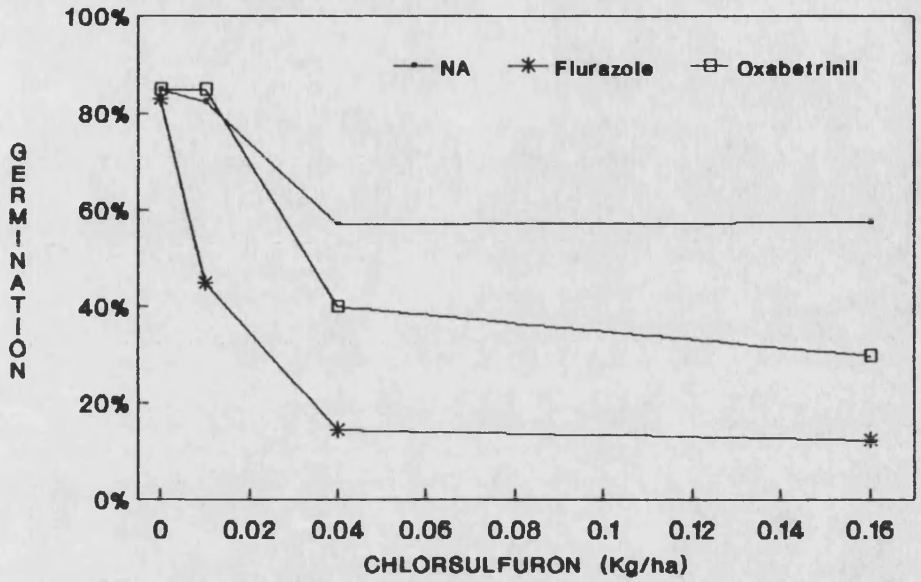
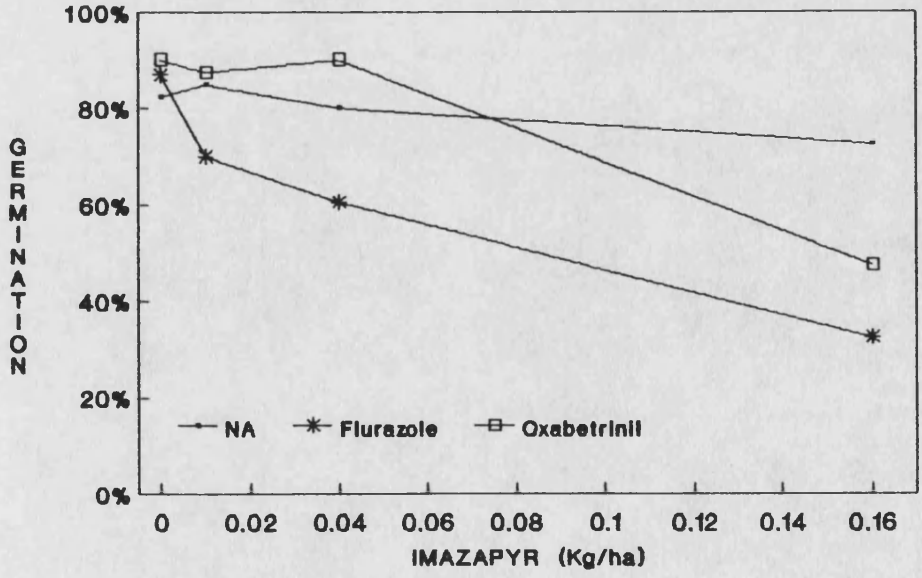


Fig. 3.6 Effect of imazapyr level on Echinochloa crus-galli germination at 13 days with each safener



The effects of chlorsulfuron and imazapyr upon germination were clearly dose dependent (Figs. 3.5 and 3.6). Anova also indicated a significant effect of safener level (P = 0.007) upon germination, but there was no clear pattern in the results (Table 3.4).

3.3.1.2 Growth effects

All of the herbicide treatments rapidly killed emerging Echinochloa crus-galli seedlings, even at the lowest rates of application. None of the safeners gave any protection to Echinochoa crus-galli against these herbicides.

Data collected from herbicide control treatments showed no significant effect of the safeners upon plant shoot fresh or dry weight, except at the highest flurazole rate (2.0%) (Tables 3.5 and 3.6), where there was a significant reduction in plant growth.

3.3.2 Experiment 1. Maize

3.3.2.1 Effect of herbicides and safeners upon germination

Unlike Echinochloa crus-galli there was no significant effect of the herbicides used upon maize germination (Fig. 3.7). There was, however, a highly significant reduction in germination with flurazole treatment in comparison to the other two safeners (Fig. 3.8).

Table 3.5 Fresh weight Echinochloa crus-galli +53 days growth with safener treatments alone

Safener	level (% seed wt.)	mean fwt/ pot (g)	S.E.(1)	no. plants	mean fwt/ plant (g)
NA	0	29.54	1.58	32	0.923
	0.25	28.35	1.92	37	0.766
	0.5	29.79	1.93	32	0.931
	1.0	31.00	1.86	37	0.838
Ox.	0	26.69	0.94	34	0.785
	0.5	28.04	1.73	38	0.738
	1.0	29.33	1.63	38	0.772
	2.0	26.61	2.39	33	0.806
Fl.	0	28.27	1.86	36	0.785
	0.5	28.10	1.91(2)	32	0.878
	1.0	27.95	1.46	39	0.717
	2.0	17.55	2.66	36	0.487

(1) n = 8

(2) n = 7

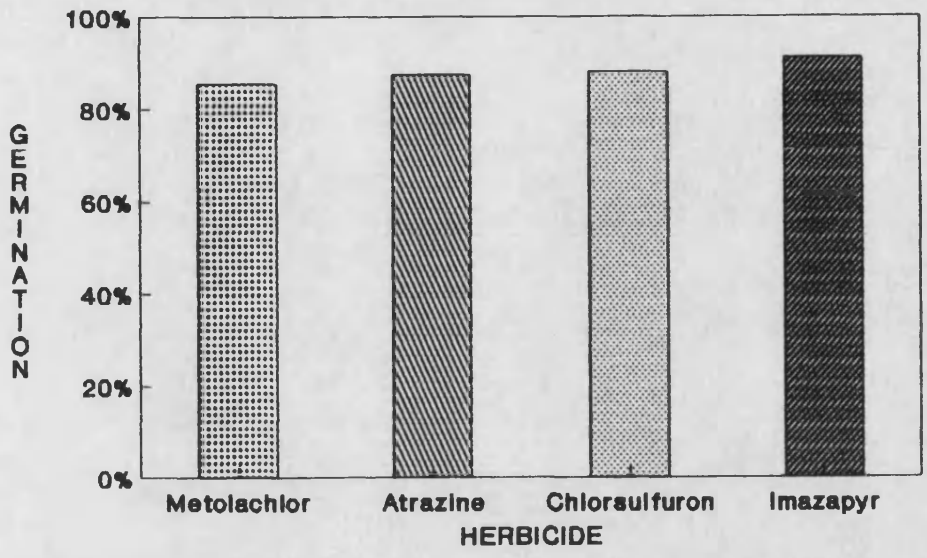
Table 3.6 Dry weight Echinochloa crus-galli +53 days growth with safener treatments alone

Safener	level (% seed wt.)	mean fwt/ pot (g)	S.E.(1)	no. plants	mean fwt/ plant (g)
NA	0	3.65	0.24	32	0.114
	0.25	3.60	0.37	37	0.097
	0.5	3.88	0.26	32	0.121
	1.0	3.75	0.28	37	0.101
Ox.	0	3.35	0.16	34	0.098
	0.5	3.58	0.38	38	0.094
	1.0	3.72	0.29	38	0.098
	2.0	3.18	0.38	33	0.096
Fl.	0	3.47	0.33	36	0.096
	0.5	3.38	0.45(2)	32	0.106
	1.0	3.18	0.28	39	0.081
	2.0	1.84	0.33	36	0.051

(1) n = 8

(2) n = 8

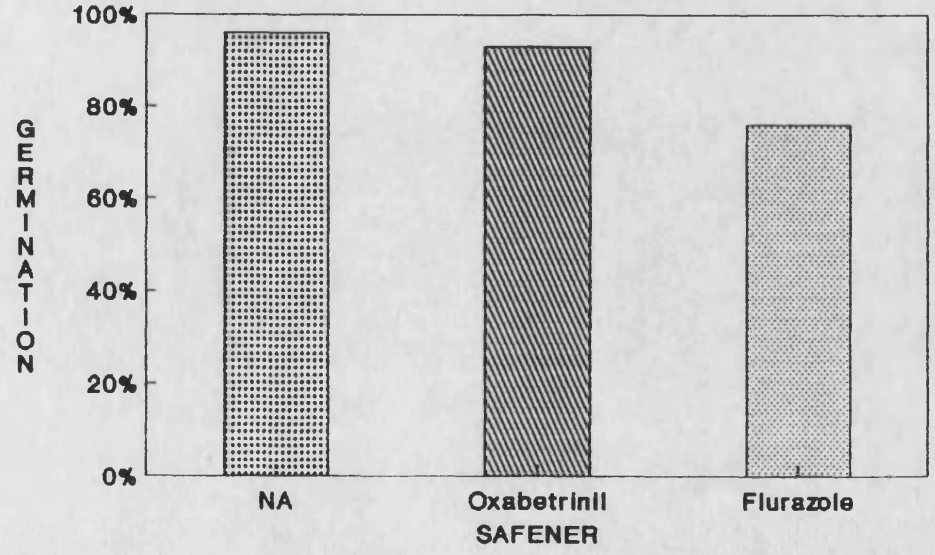
Fig. 3.7 Effect of herbicides on Zea mays var. LG 11 germination 10 days after sowing



anova: Fpr. = 0.370

control germination = 94%

Fig. 3.8. Effect of safeners on Zea mays var. LG 11 germination 10 days after sowing



anova: Fpr. < 0.001

control germination = 94%

Table 3.7 Germination of maize with herbicide x safener treatments

	% germination(1)		
Safener	NA	Ox	Fl.
Herbicide			
Met.	98.4	95.3	62.5
Atr.	92.2	92.2	78.1
Chl.	98.4	89.1	78.1
Ima.	95.3	93.8	84.4

anova Fpr. = 0.017

(1) Average of 16 values

Control germination = 94%

Anova of individual herbicide x safener combinations indicated a significant (P = 0.017) herbicide x safener interaction effect. The low metolachlor x flurazole value (Table 3.7) suggested an interaction effect between this herbicide and safener.

Comparison of germination rates with the safener flurazole for each of the herbicide treatments revealed that the inhibitory effect of flurazole upon germination was dose dependent (Fig. 3.9).

3.2.2.2 Growth effects

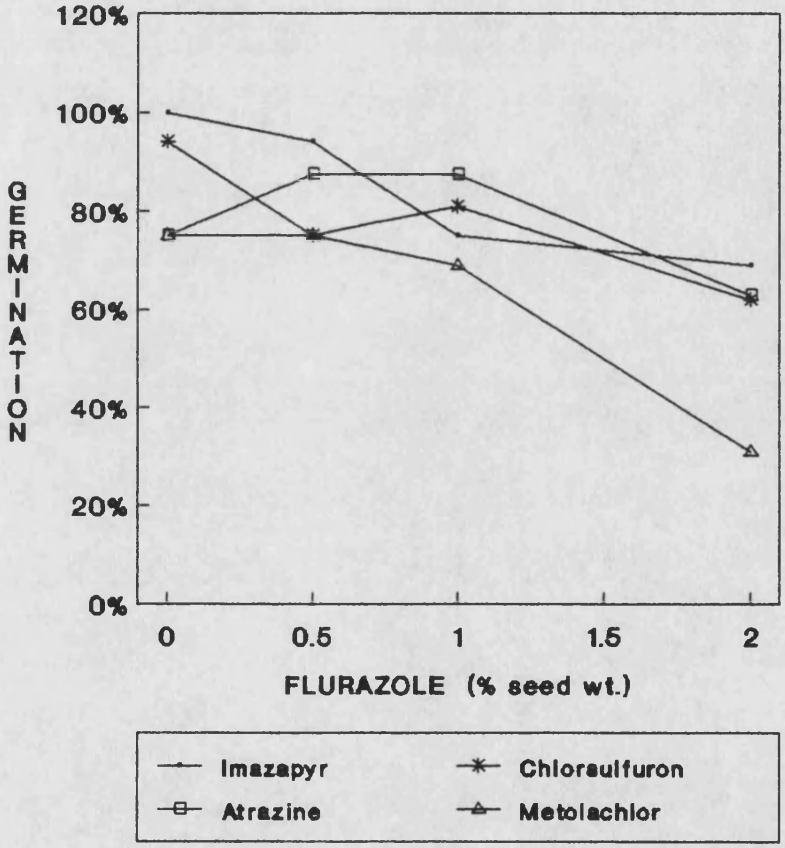
(i) Safening effects against herbicide action

A summary of anova results for maize shoot fresh and dry weights (appendix 1) and leaf extension data (appendix 2) revealed no significant effect of either metolachlor or atrazine upon maize growth at these rates of application. No visual effects were observable during the growth period or at harvest.

Both chlorsulfuron and imazapyr had highly significant effects ($P < 0.001$) upon maize growth (appendix 1 and 2). Plates 3.1-3.4 show that both herbicides caused a severe stunting of shoot growth that was dose dependent.

None of the safeners gave any visible protection to maize against imazapyr, as is shown by the example of imazapyr x NA treatments in plate 3.4. Anova of fresh and dry weight values of plant shoots at

Fig. 3.9 Effect of flurazole level on Zea mays var. LG 11 germination at 10 days with each herbicide



anova: Fpr. = 0.002

Plate 3.1 Effect of chlorsulfuron and NA treatments on maize +24 days growth.

Plate 3.2 Effect of chlorsulfuron and oxabetrinil treatments on maize +24 days growth.


CHL.
0
CHL.
0.01 kg ha⁻¹
CHL.
0.06 kg ha⁻¹
CHL.
0.16 kg ha⁻¹
NA
0
NA
0.25 %
NA
0.5 %
NA
1.0 %

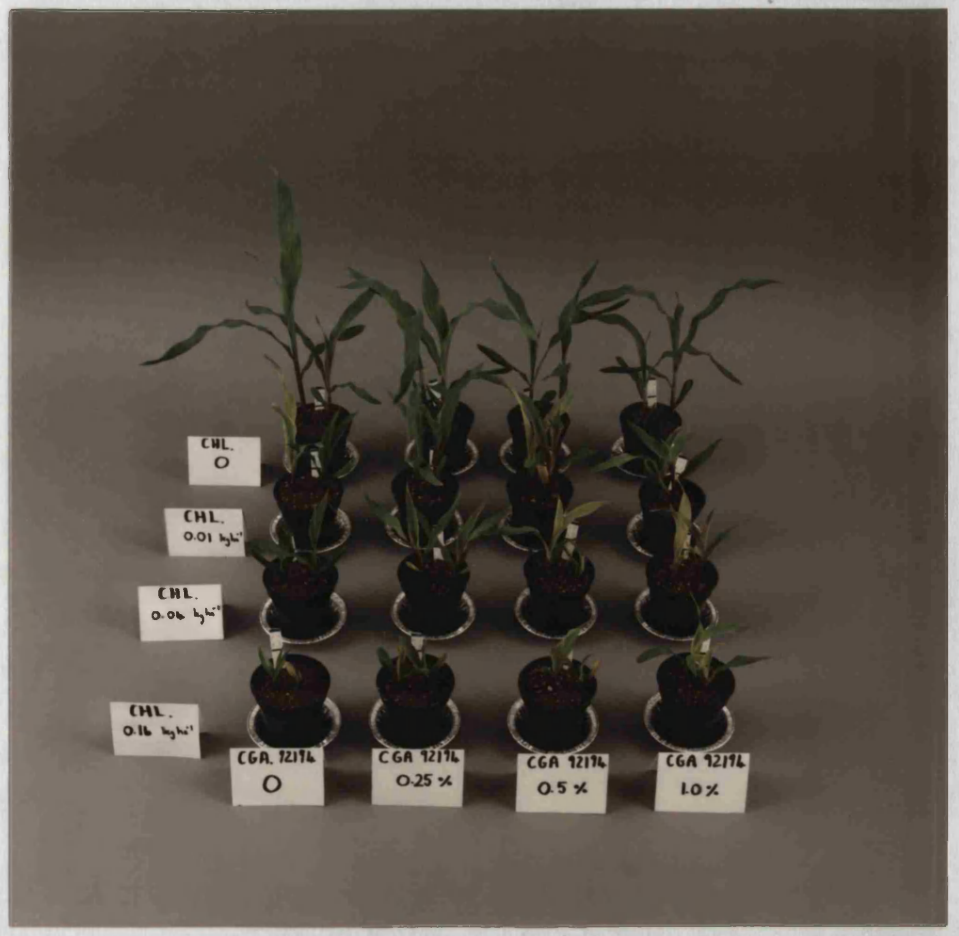
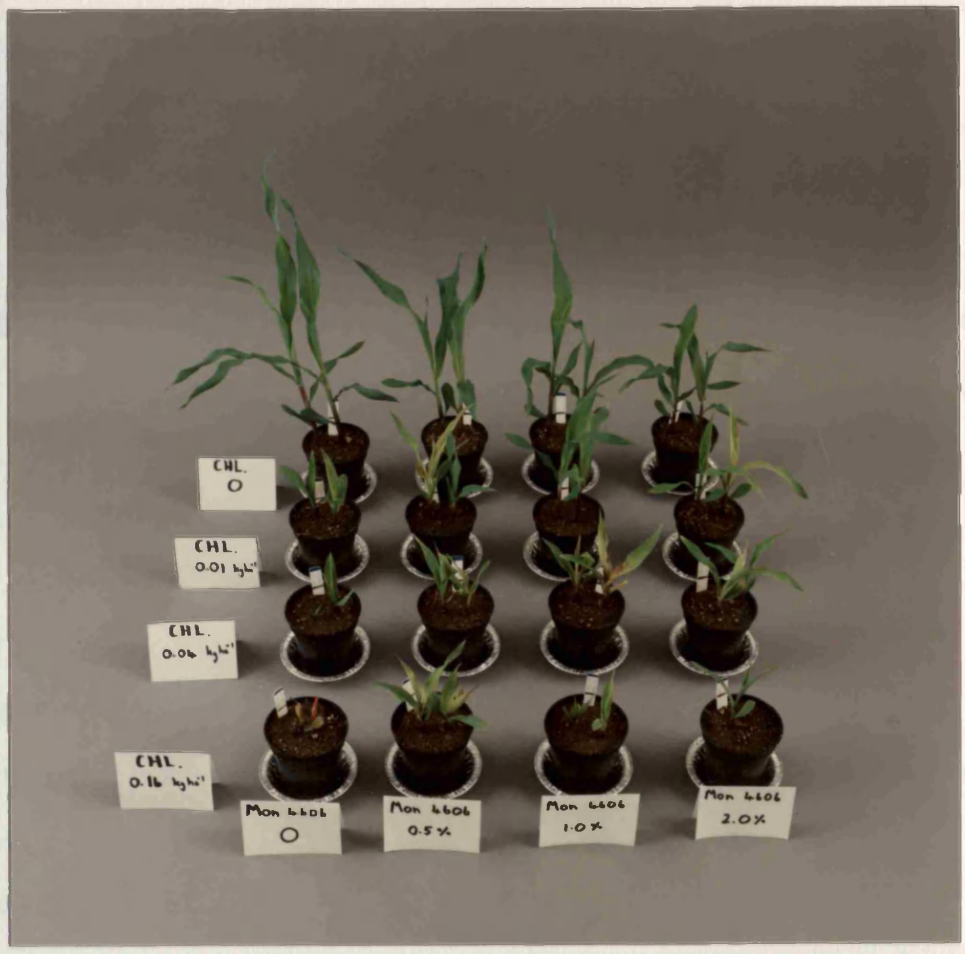
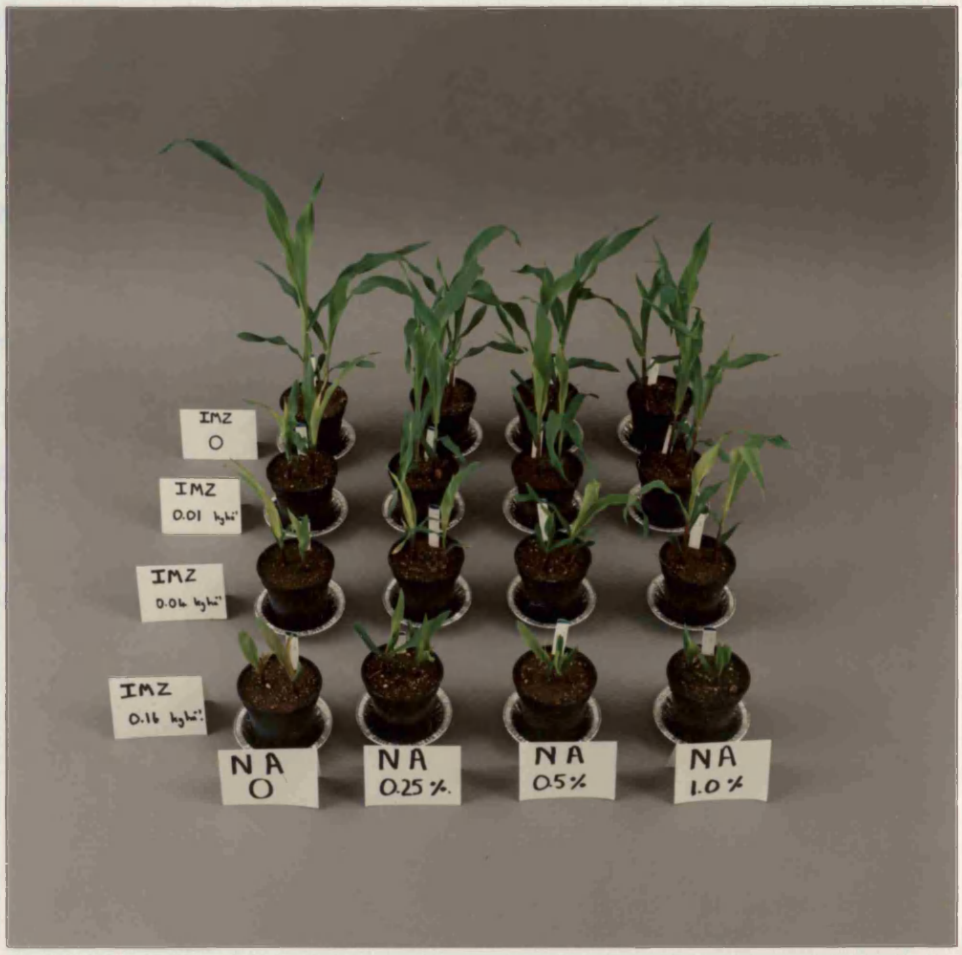

CHL.
0
CHL.
0.01 kg ha⁻¹
CHL.
0.06 kg ha⁻¹
CHL.
0.16 kg ha⁻¹
CGA 72174
0
CGA 72174
0.25 %
CGA 72174
0.5 %
CGA 72174
1.0 %

Plate 3.3 Effect of chlorsulfuron and flurazole treatments on maize +24 days growth.

Plate 3.4 Effect of imazapyr and NA treatments on maize +24 days growth.


CHL.
0
CHL.
0.01 kghi⁻¹
CHL.
0.06 kghi⁻¹
CHL.
0.16 kghi⁻¹
Mon 4606
0
Mon 4606
0.5%
Mon 4606
1.0%
Mon 4606
2.0%


IMZ
0
IMZ
0.01 kghi⁻¹
IMZ
0.06 kghi⁻¹
IMZ
0.16 kghi⁻¹
NA
0
NA
0.25%
NA
0.5%
NA
1.0%

34 days growth (appendix 1) also gave no indication of any safener effect, or interaction with imazapyr.

Visually, NA, oxabetrinil and flurazole all gave some protection to maize against the stunting effect of chlorsulfuron (Plates 3.1, 3.2 and 3.3). NA appeared to give marginally better protection than oxabetrinil or flurazole.

Anova of shoot fresh and dry weights, and third leaf length revealed a highly significant effect ($P < 0.001$) of NA level upon growth with chlorsulfuron (appendix 1 and 2). Figures 3.10 and 3.11 show that NA protection was only partial. There was a slight enhancement of protective action with increasing NA application rates at a given herbicide level, and this response was more obvious at the highest rate of chlorsulfuron. Leaf length data indicated a significant herbicide level x safener level interaction effect ($P = 0.05$ to 0.01), but this was not reflected in fresh and dry weight data (appendix 1 and 2).

Oxabetrinil gave less protection to maize against chlorsulfuron than NA, but still had a significant effect ($0.001 < P < 0.05$) on fresh and dry weights and leaf length (appendix 1 and 2). There was no indication of an increase in safening effect with oxabetrinil application rate, except at the highest chlorsulfuron level (Figs. 3.12 and 3.13).

FIG. 3.10 a) Effect of chlorsulfuron and NA on Zea mays var. LG 11 fresh weight + 34 days growth

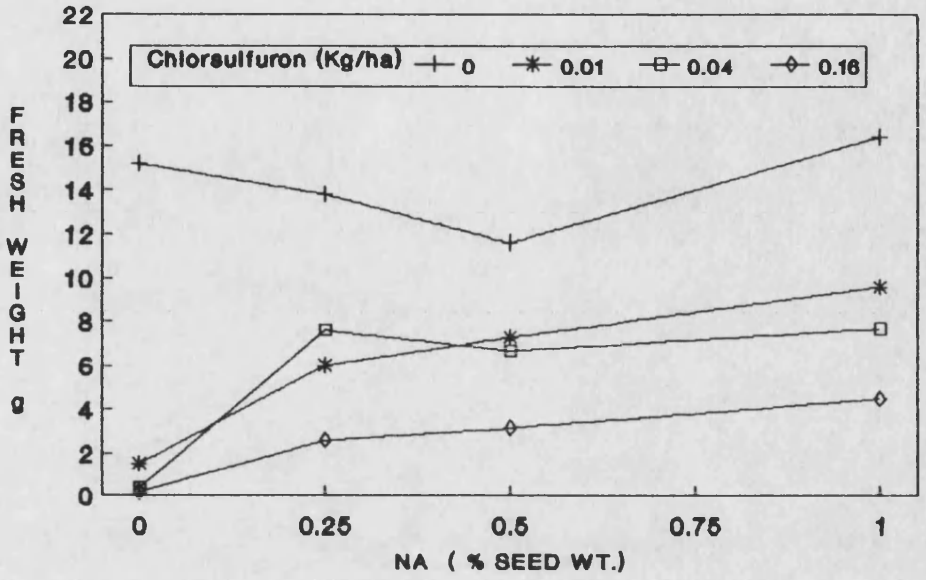


Fig. 310 b) Effect of chlorsulfuron and NA on Zea mays var. LG 11 dry weight +34 days growth

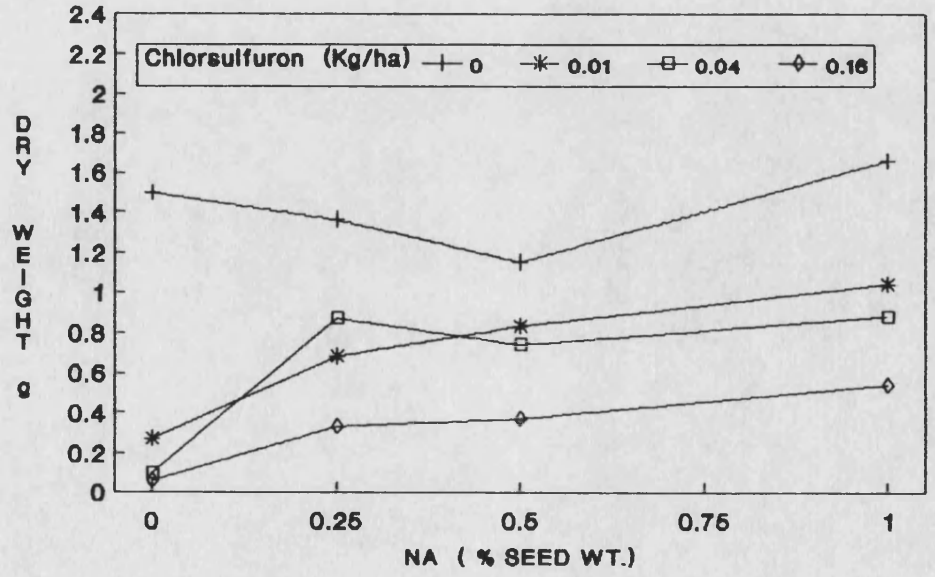
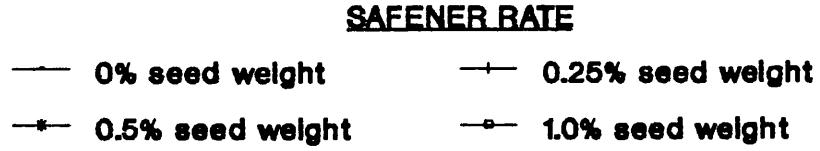
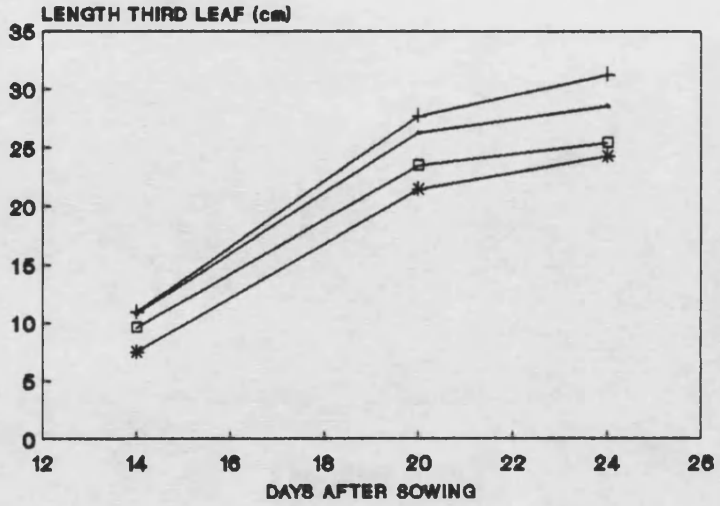


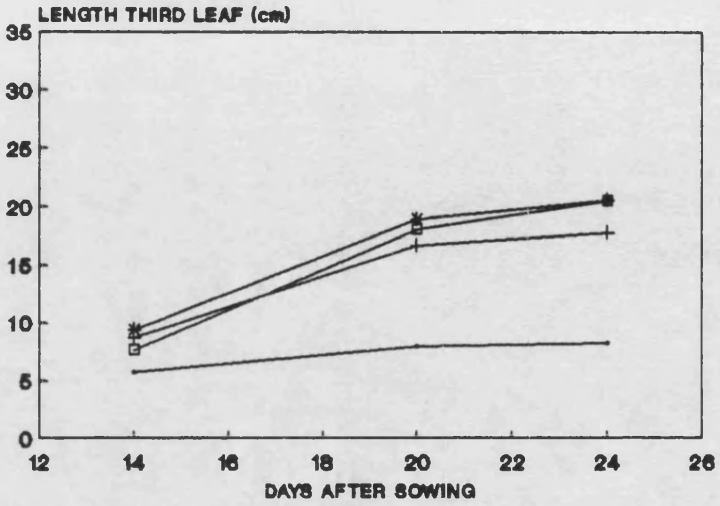
Fig. 3.11 The effect of NA and chlorsulfuron on Zea mays var. LG 11 leaf growth



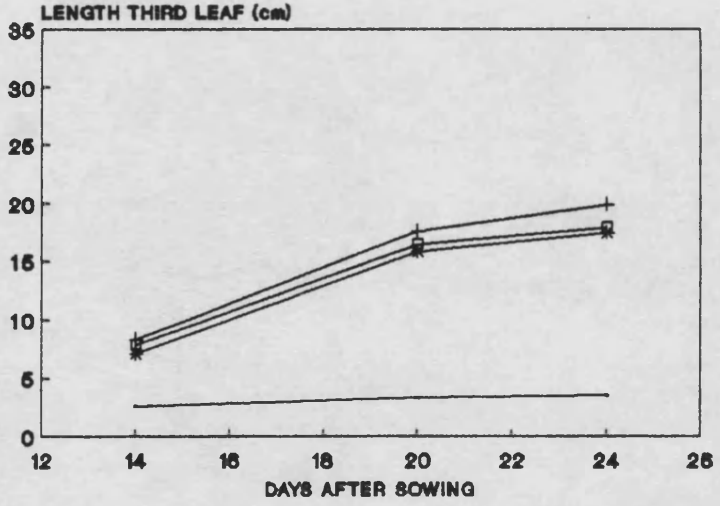
a) 0 Kg/ha chlorsulfuron



b) 0.01 Kg/ha chlorsulfuron



c) 0.04 Kg/ha chlorsulfuron



d) 0.16 Kg/ha chlorsulfuron

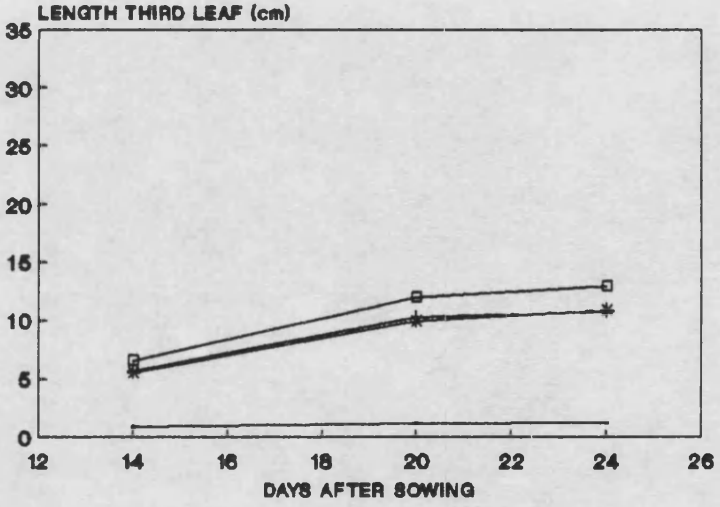


Fig. 3.12 a) Effect of chlorsulfuron and oxabetrinil on Zea mays var LG 11 fresh weight + 34 days growth

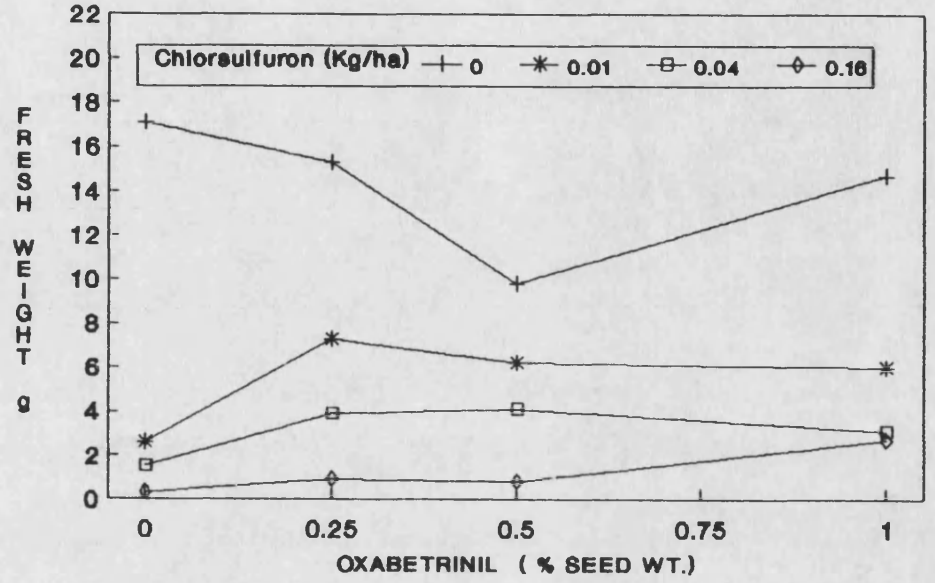


Fig. 3.12 b) Effect of chlorsulfuron and oxabetrinil on Zea mays var. LG 11 dry weight + 34 days growth

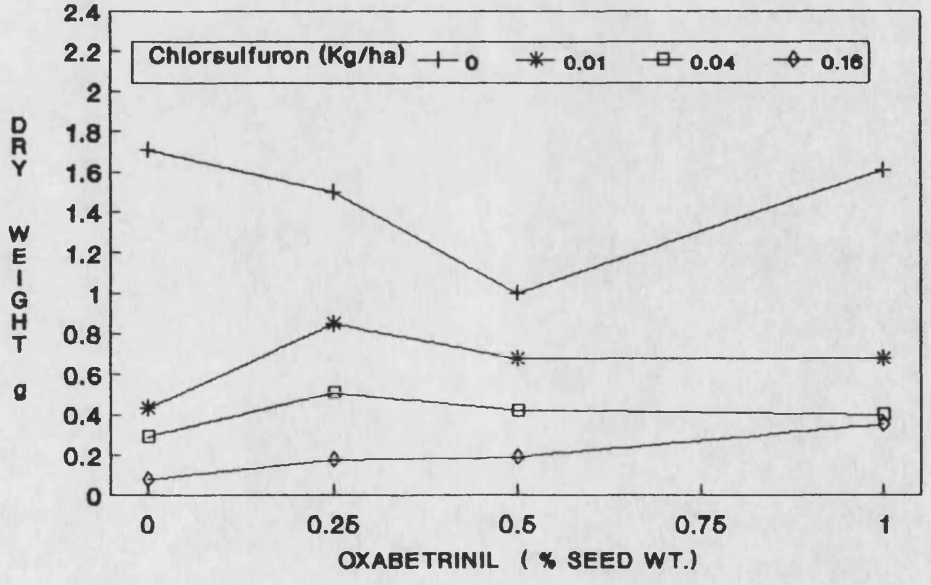
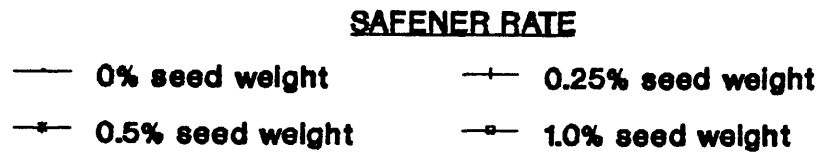
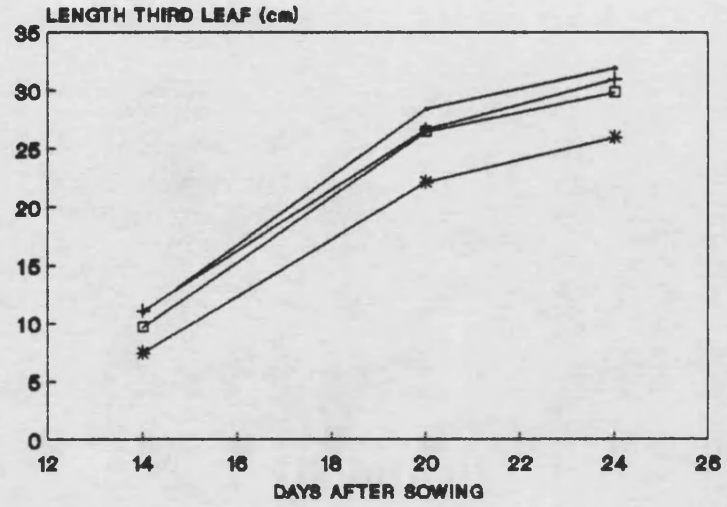


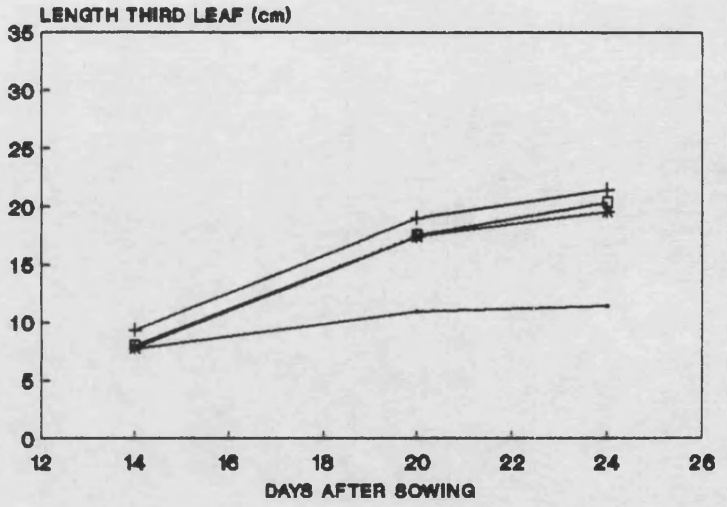
Fig. 3.13 The effect of oxabetrinil and chlorsulfuron on Zea mays var. LG 11 leaf growth



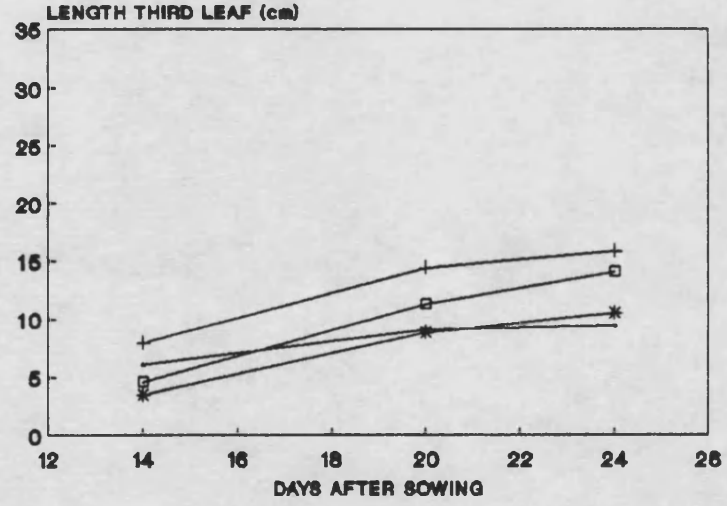
a) 0 Kg/ha chlorsulfuron



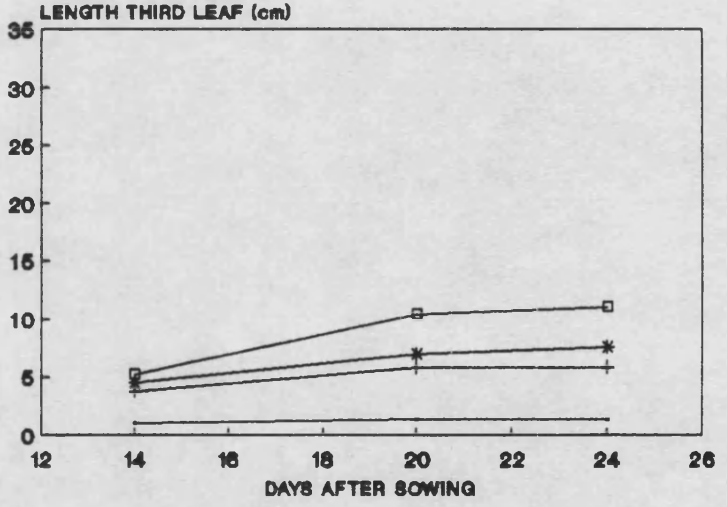
b) 0.01 Kg/ha chlorsulfuron



c) 0.04 Kg/ha chlorsulfuron



d) 0.16 Kg/ha chlorsulfuron



Fresh and dry weight data gave no indication of a significant effect of flurazole rate upon maize response to chlorsulfuron (appendix 1). Leaf length also gave no significant effect except at the final measurement (appendix 2). Fresh and dry weights and leaf length data did give a significant herbicide level x safener level interaction. Figures 3.14 and 3.15 show some protective effect of flurazole against chlorsulfuron. There was no clear indication of an increase in protective action with safener rate, but there was a reduction in growth of non-herbicide treated plants with safener application.

(ii) Stunting Effects Associated with Safener Treatment

Visual assessment of herbicide x safener treatments indicated a slight stunting of plant growth associated with safener application in herbicide free treatments (e.g. Plates 3.1-3.4). Leaf extension data for the herbicide free treatments showed partial inhibition of growth associated with all three safeners, which increased with safener application rate (Figs. 3.16, 3.17 and 3.18).

Some significant effects of safeners upon leaf length occurred at all three sample times with the non-safened herbicides atrazine, metolachlor and imazapyr (appendix 2). Leaf mean length values indicated a reduction in leaf length associated with increasing safener application rates (appendix 3). However, significant safener effects were not always consistent across the three sample times, and only the metolachlor x flurazole treatment showed any

Fig. 3.14 a) Effect of chlorsulfuron and flurazole on Zea mays var. LG 11 fresh weight + 34 days growth

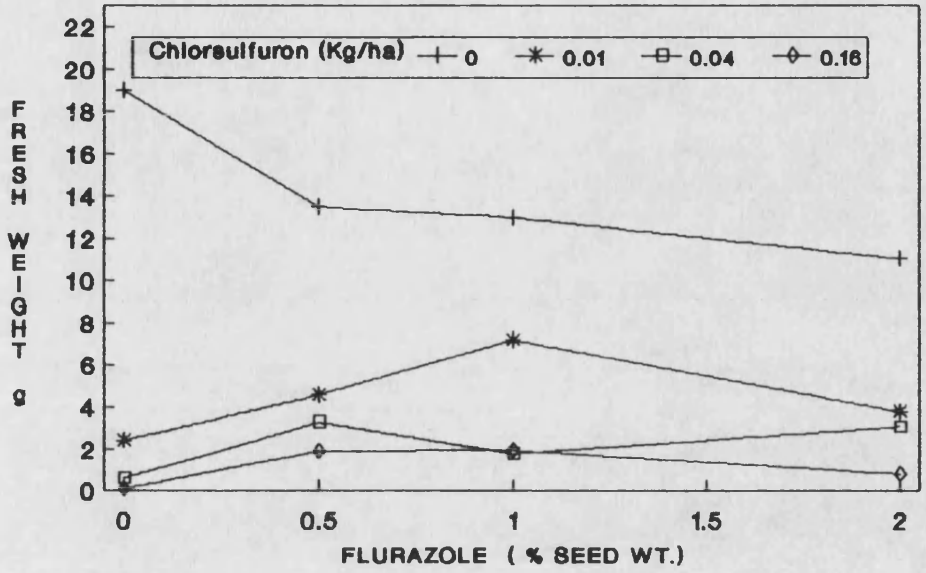


Fig. 3.14 b) Effect of chlorsulfuron and flurazole on Zea mays var. LG 11 dry weight + 34 days growth

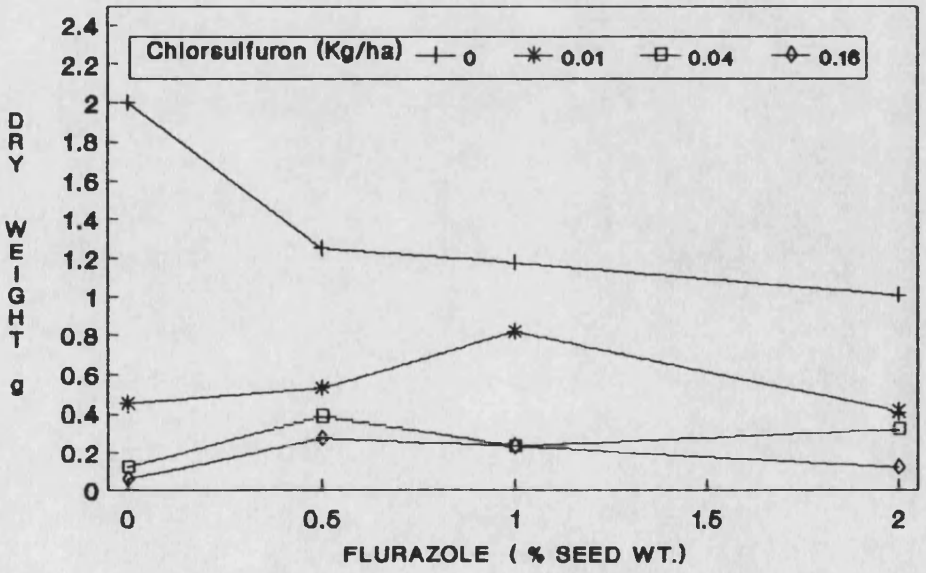
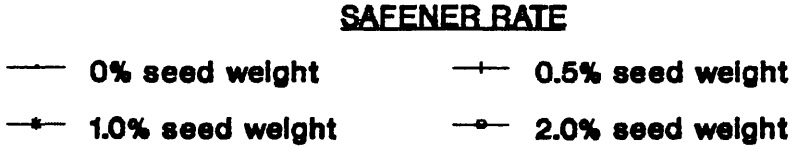
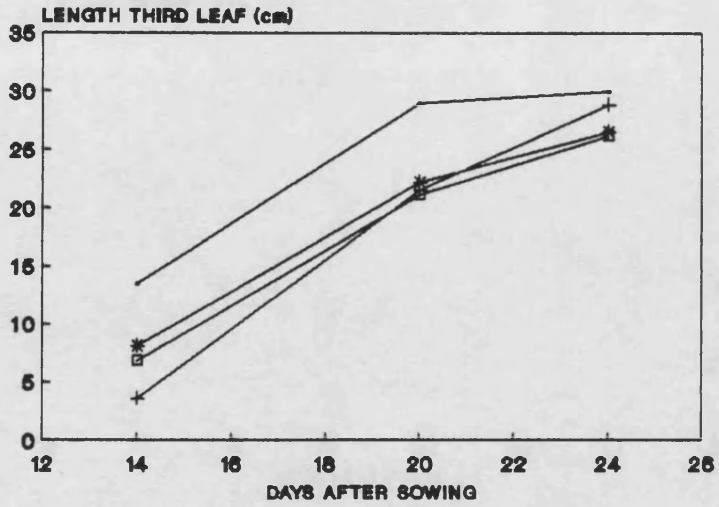


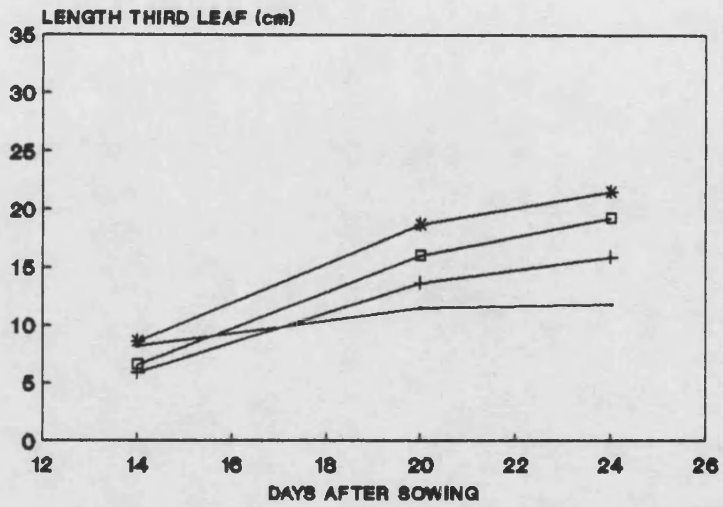
Fig. 3.15 The effect of flurazole and chlorsulfuron on Zea mays var. LG 11 leaf growth



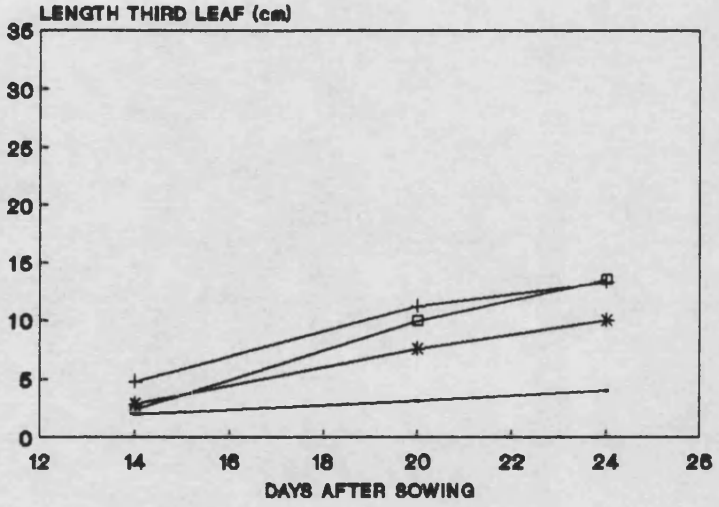
a) 0 Kg/ha chlorsulfuron



b) 0.01 Kg/ha chlorsulfuron



c) 0.04 Kg/ha chlorsulfuron



d) 0.16 Kg/ha chlorsulfuron

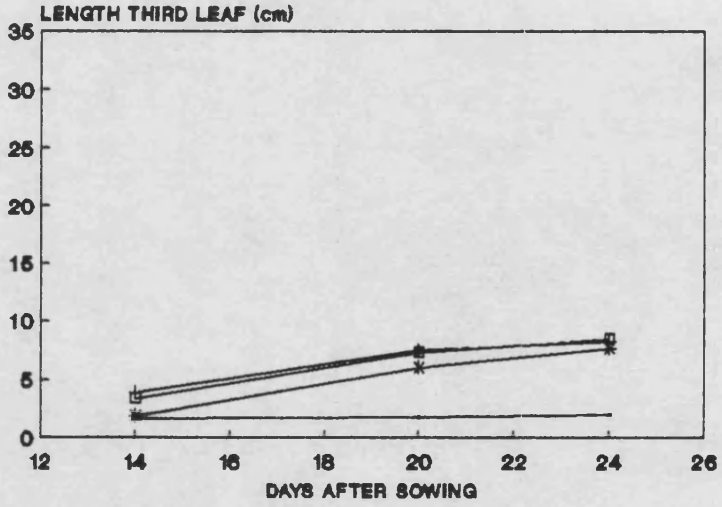


Fig. 3.16 Effect of NA on Zea mays var. LG 11 third leaf growth in absence of herbicide treatment

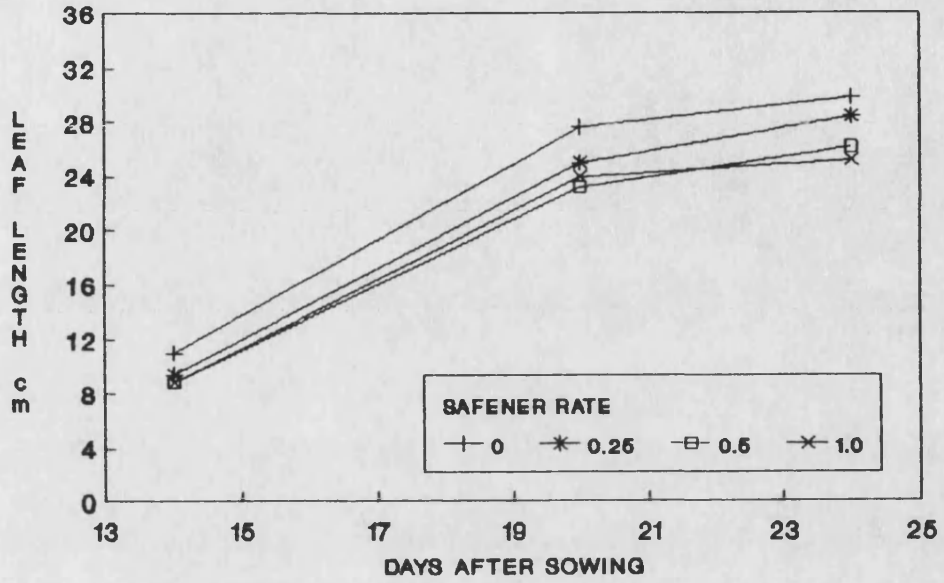


Fig. 3.17 Effect of oxabetrinil on Zea mays var. LG 11 third leaf growth in absence of herbicide treatment

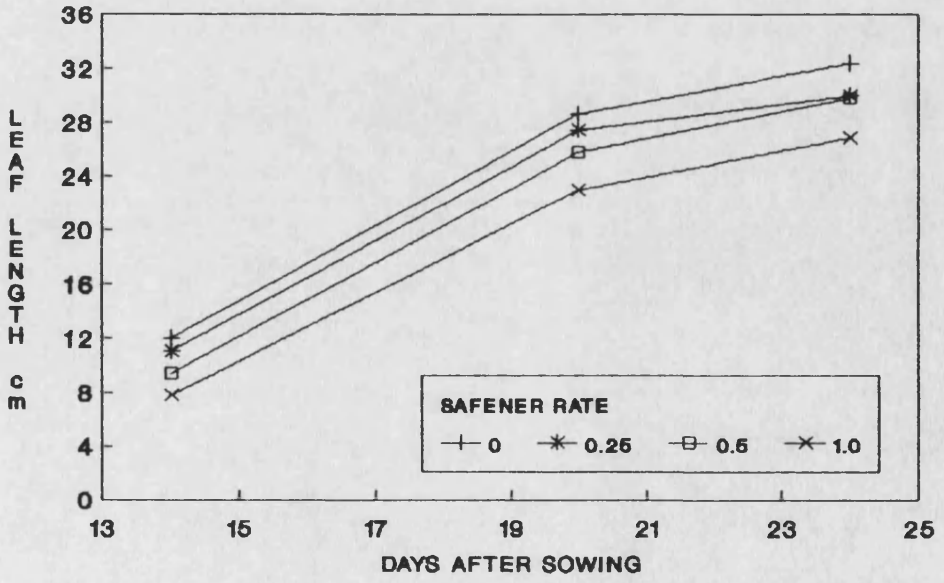
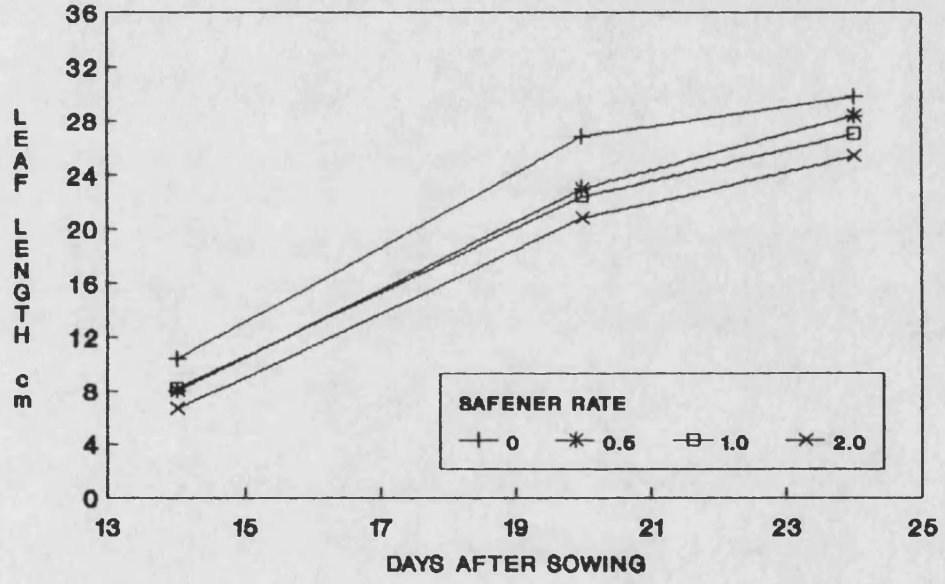


Fig. 3.18 Effect of flurazole on
Zea mays var. LG 11 third leaf growth
in absence of herbicide treatment



significant reduction in shoot fresh or dry weight associated with safener treatment (appendix 1 and Table 3.8).

3.3.3 Experiment 2. Maize x chlorsulfuron x dichlormid

Neither chlorsulfuron nor dichlormid had any effect upon maize germination rates. Germination at 10 days after sowing for the whole trial was 99.4%.

All levels of dichlormid provided some protection against chlorsulfuron damage to maize (Plate 3.5, Figs. 3.19 and 3.20). The effects of dichlormid level, and chlorsulfuron level x dichlormid level were highly significant ($P < 0.001$) for fresh and dry weights, and leaf length data. There was a slight increase in safening response with safener level for most of the herbicide x safener combinations (Fig. 3.19). At the lowest chlorsulfuron rate (0.01 kg/ha) dichlormid at 0.5% and above gave almost complete protection against chlorsulfuron damage, as measured by fresh and dry weight data.

Dichlormid treatments caused no significant reduction in plant fresh and dry weights in herbicide free controls (Fig. 3.19), but there was an indication of a slight reduction in leaf length associated with dichlormid application at each sample date (Fig. 3.20).

Table 3.8 Effect of metolachlor and flurazole levels on maize fresh and dry weights

a) Fresh weights (g)(1)

Herbicide level (kg/ha)	Safener level (% seed wt) 0	0.5	1.0	2.0	$\bar{x}$
0	17.73	12.02	10.69	11.69	13.03
0.25	21.07	13.22	8.44	2.65	11.34
1.0	14.77	8.54	13.97	8.28	11.39
4.0	9.03	16.62	16.83	12.11	13.64
$\bar{x}$	15.65	12.60	12.48	8.68	

b) Dry weights (g)(1)

Herbicide level (kg/ha)	Safener level (% seed wt) 0	0.5	1.0	2.0	$\bar{x}$
0	1.815	1.130	0.905	0.928	1.195
0.25	2.110	1.225	0.770	0.195	1.075
1.0	1.475	0.760	1.228	0.710	1.043
4.0	0.875	1.625	1.508	1.058	1.267
$\bar{x}$	1.569	1.185	1.103	0.723	

(1) Average of 2 values

Plate 3.5 Effect of chlorsulfuron and dichlormid treatments on maize +23 days growth.


chl.
0
chl.
0.01
chl.
0.04
R25788
0
R25788
025%
R25788
0.5%
R25788
1.0%

FIG. 3.19 a) Effect of chlorsulfuron and dichlormid on Zea mays var. LG 11 fresh weight + 25 days growth

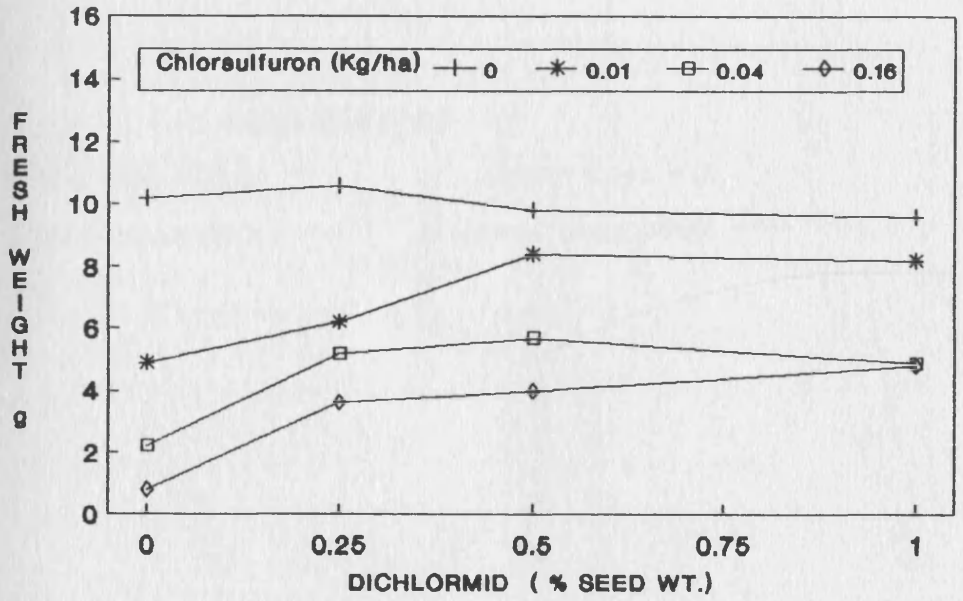


FIG. 3.19 b) Effect of chlorsulfuron and dichlormid on Zea mays var. LG 11 dry weight + 25 days growth

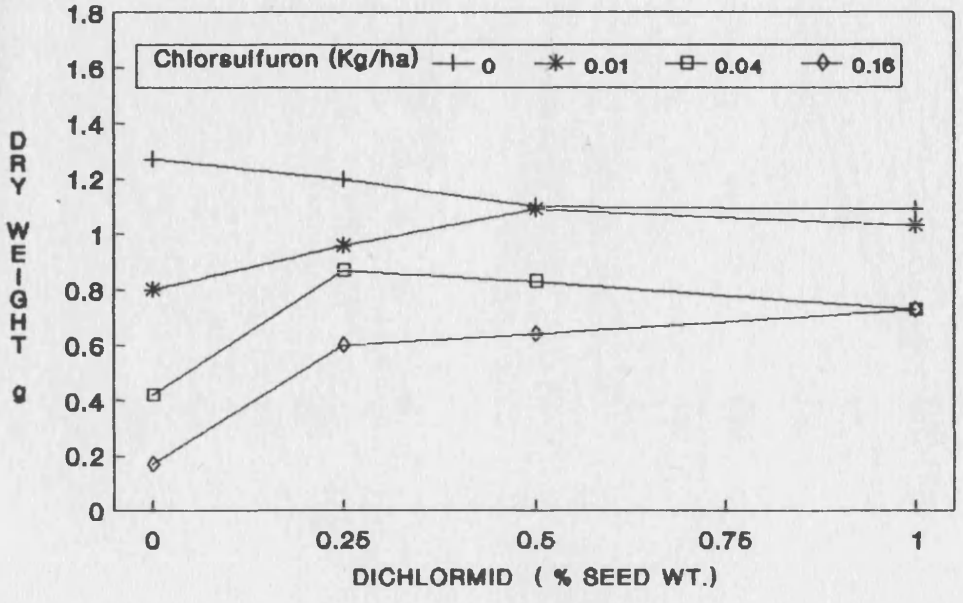
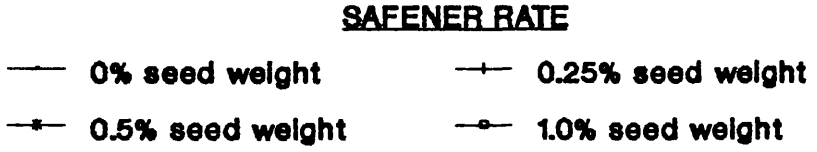
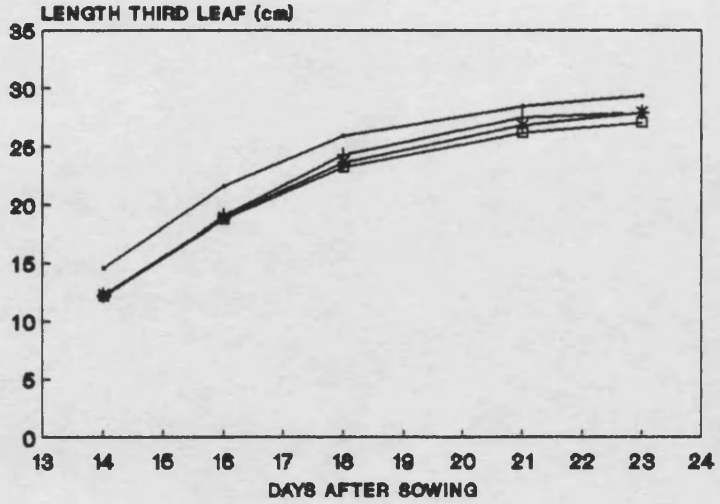


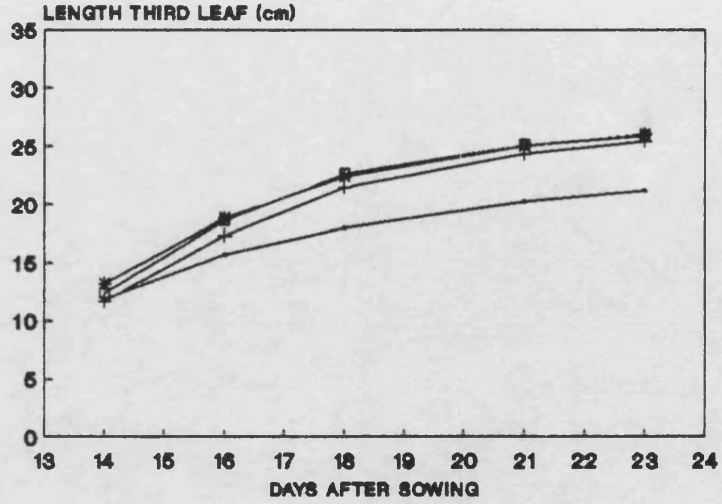
Fig. 3.20 The effect of dichlormid and chlorsulfuron on Zea mays var. LG 11 leaf growth



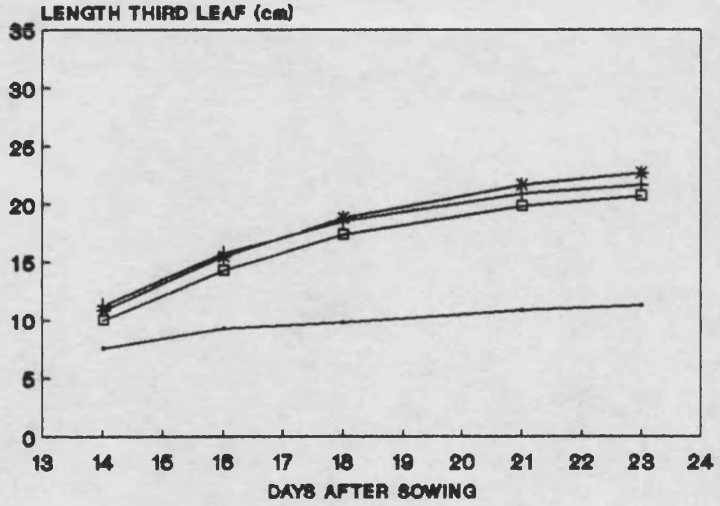
a) 0 Kg/ha chlorsulfuron



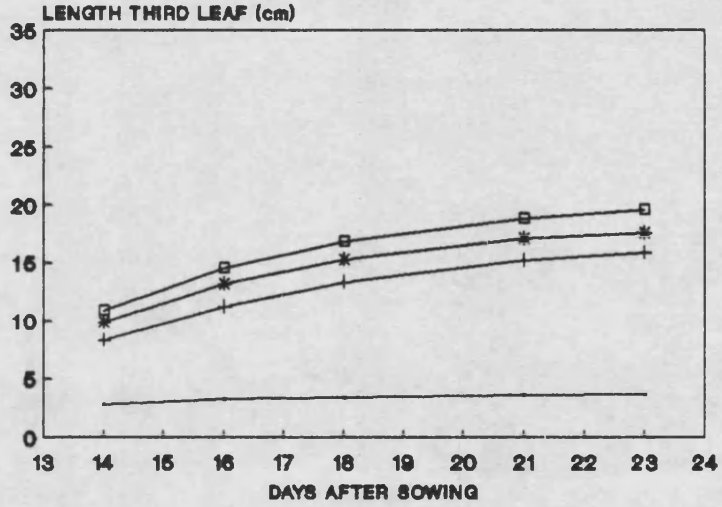
b) 0.01 Kg/ha chlorsulfuron



c) 0.04 Kg/ha chlorsulfuron



d) 0.16 Kg/ha chlorsulfuron



3.4 DISCUSSION

The safeners NA, oxabetrinil and flurazole did not give any protection to Echinochloa crus-galli against pre-emergence applications of either atrazine, chlorsulfuron, imazapyr or metolachlor. These results seem consistent with the few reports in the literature of safener action with this species. Rubin, Kirino and Casida (1985) reported slight protection of Echinochloa crus-galli against pre-emergence application of alachlor by flurazole. However, Schafer, Brinker and Radke (1980) found no safening of Echinochloa crus-galli against preplant incorporated alachlor by flurazole. Cyometrinil gave minimal protection to Echinochloa crus-galli against metolachlor (Nyffeler, Gerber and Hensley, 1980), whereas oxabetrinil and fenclorim gave no protection against pre-emergence applications of metolachlor (0.25 kg/ha) and pretilachlor (1.0 kg/ha) (Ebert and Gerber, 1989).

Neither metolachlor nor atrazine caused any measurable effects on maize growth, or visible symptoms at the rates applied in the experiment, hence no safening response was observable. NA has been reported to provide partial protection to maize against some of the chloroacetanilides, but not metolachlor (Leavitt and Penner, 1978), and flurazole to metolachlor and alachlor (Hatzios, 1982). No success in safening against triazine toxicity has been reported (Hatzios, 1983c). It would be interesting to ascertain if higher rates of atrazine caused toxic symptoms which could be alleviated by safener treatment, as glutathione-s-transferases are involved in

triazine metabolism in maize (Hatzios and Penner, 1982), and evidence for safener enhancement of this system is good (Section 2.3.3).

NA, oxabetrinil, flurazole and dichlormid all provided partial protection against the stunting effects caused by chlorsulfuron in maize, but neither NA, oxabetrinil nor flurazole gave any measurable protection against imazapyr. Dichlormid and NA provided better protection to maize than did flurazole or oxabetrinil. There was some evidence for increased safener protection with application rate, especially at the highest rate of chlorsulfuron application.

A number of previous reports have been made of safener activity against chlorsulfuron and other sulfonylureas in maize. NA has been reported to provide some protection (Parker, Richardson and West, 1980; Hatzios, 1984b; Rubin and Casida, 1985; O'Leary and Prendeville, 1985) as has dichlormid (Parker, Richardson and West, 1980; Hatzios, 1984b) and cyometrinil and oxabetrinil (Hatzios, 1984b). Parker, Richardson and West (1980) and Hatzios (1984b) found that NA gave better protection than dichlormid, and Hatzios (1984b) found cyometrinil and oxabetrinil protection to be intermediate between these two. The results of this trial however, showed the level of dichlormid protection to be as good as that provided by NA on this variety of maize. Oxabetrinil provided much poorer protection. NA has been reported to provide partial protection against another sulfonylurea, metsulfuron-methyl in maize (Mersie and Foy, 1984; Richardson, West and White, 1984.)

NA, dichlormid and cyometrinil have also been found to reduce the half-life of chlorsulfuron and metsulfuron-methyl in treated maize leaves (Sweetser, 1985).

Both the imidazolines and sulfonylureas reduce plant growth by inhibiting the activity of the enzyme acetohydroxyacid synthetase (AHAS) that is involved in the production of the branch chained amino acids valine leucine and isoleucine (Hawkes, Howard and Pontin, 1989). NA, oxabetrinil and flurazole have been reported to provide partial protection to maize from pre-emergence applications of two imidazolinones; imazaquin and imazethapyr (Barrett and Olson, 1986; Barrett, 1988). However, this trial showed no protective action with these safeners against imazmapyr with this maize variety. It is interesting to note that imazapyr is the least selective of the imidazolinone group of herbicides, and so any safening response would be particularly useful in extending its range of application.

Some phytotoxic effects associated with safener treatment were apparent for all the compounds used. NA, oxabetrinil, flurazole and dichlormid all caused a slight visual stunting effect on herbicide free treatments in maize, and this was reflected in a slight inhibition in the growth of the third leaf of treated plants. Some inhibition of leaf growth was also apparent for safener treatments in combination with atrazine, metolachlor and imazapyr where no "safening effect" was observed. Reductions in plant growth with safener treatments were not apparent from plant fresh and dry

weights for either maize or Echinochloa crus-galli, except with flurazole.

Flurazole caused a significant reduction in seed germination rates for both maize and Echinochloa crus-galli. Neither NA nor oxabetrinil had any significant effect on these two species, or dichlormid on maize. The effect of flurazole on the germination of maize and Echinochloa crus-galli was contrary to that found by Schafer, Brinker and Radke (1980, 1981) in sorghum, but Ketchersid and Merkle (1983) found measurable effects of flurazole on the respiration and early growth of sorghum seedlings. Besides the species difference, it should be noted that very high flurazole and oxabetrinil application rates were used in this trial. Rates of 0.5–2.0% and 0.25–1.0% (0.5–2.0% for Echinochloa crus-galli) were used for flurazole and oxabetrinil respectively, compared with advised application rates for sorghum of 0.125% and 0.05–0.3% by seed weight respectively (Hatzios, 1983c).

4. THE EFFECT OF NA AND DICHLORMID ON GLUTATHIONE AND GLUTATHIONE-S-TRANSFERASE LEVELS IN MAIZE

4.1 INTRODUCTION

As was discussed in Section 2.3.3, glutathione conjugation has been reported to be an important step in the metabolism of several types of herbicides in plants, including the thiocarbamates (Hatzios and Penner, 1982). This reaction is believed to be mediated by glutathione-s-transferases, but has been found to occur non-enzymatically at low rates under physiological conditions (Ezra et al., 1985; Gronwald et al., 1987; Leavitt and Penner, 1979).

Both NA and dichlormid provide good protection to maize against the thiocarbamate herbicide EPTC (Hatzios, 1983c; Parker, 1983). There is good evidence to indicate that dichlormid enhances the level of GSH and GSTs in maize (Carringer, Rieck and Bush, 1978; Lay, Hubbel and Casida, 1975; Lay and Niland, 1985; Stephenson, Ali and Ashton, 1983), and that this response is correlated to the ability of the safener to protect against EPTC damage (Stephenson, Ali and Ashton, 1983).

The evidence for an enhancement of glutathione conjugation being involved in the protective action of NA is less certain. Lay and Casida (1976) and Fedtke (1981) found no effect of NA upon the GSH content of maize roots. Lay and Casida (1976) also reported no effect of NA on GST activity in maize, however, other workers have

found an enhancement of GST activity following NA application (Fedtke, 1981; Kömives et al., 1985b; Mozer, Tiemeier and Jaworski, 1983). NA has also been found to elevate the activity of GST in sorghum plants (Gronwald et al., 1987).

As an initial investigation of some of the biochemical effects associated with safener action, and in particular the role of glutathione and GST levels in modulating herbicide activity, a comparison of the effects of NA and dichlormid on glutathione and GST levels in maize was made.

4.2 MATERIALS AND METHODS

4.2.1 Plant Material and Growth Conditions

Seeds of maize (var. LG11) were washed for 15 minutes in running tap water and dried, in order to remove a fungicide dressing of captan and thiram. Either NA (97% w/w ai) or dichlormid (20% w/w ai) was applied to the washed seeds at rates of 0, 0.25, 0.5 and 1.0% by seed weight, as described in Section 3.2.3.

Three seeds were sown per 9 cm pot in vermiculite, and watered thoroughly with 50% Hoaglands solution (Hoagland and Arnon, 1950). Pots were placed in a cabinet under fluorescent lighting with a 16 hour photoperiod at 390 $\mu EM^{-2}s^{-1}$, and day/night temperatures of 27/19°C respectively. All pots were watered every two days with 50 ml of nutrient solution.

4.2.2 Glutathione, GST and Protein Assays

All assays were carried out using root and shoot tissue from plants five days after sowing, which were at the two leaf stage.

(i) Assay of total glutathione (GSH + GSSG)

The assay procedure used was a modification of methods described by Law, Charles and Halliwell (1983) and Smith et al. (1984).

Glutathione was sequentially oxidised by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and reduced by NADPH in the presence of glutathione reductase (Fig. 4.1). A linear rate of DTNB reduction was observed for samples containing more than 0.5 nM glutathione throughout the assay. Accumulation of reduced DTNB was measured spectrophotometrically as an increase in absorption at 412 nm.

Approximately 0.25 g (fresh weight) of root or shoot tissue was ground in a glass homogeniser in 10 ml of 0.5% (w/v) sulphosalycylic acid on ice. 2-3 ml was poured into a centrifuge tube and spun at 4000 rpm in a benchtop centrifuge for 10 minutes at 4°C. 375 µl of the resulting supernatant was added to 875 µl 0.6 mM NADPH, 875 µl 125 mM NaH_2PO_4 buffer containing 6.3 mM EDTA at pH 7.5, 250 µl 6 mM DTNB and 125 µl glutathione reductase at 10 units/ml. All reagents were made up in NaH_2PO_4/EDTA buffer, pH 7.5. The assay was carried out at 25°C, and the $[A_{412}]$nm recorded using a Shimadzu UV-260 UV-VIS spectrophotometer.

Fig. 4.1 Reaction scheme for glutathione assay

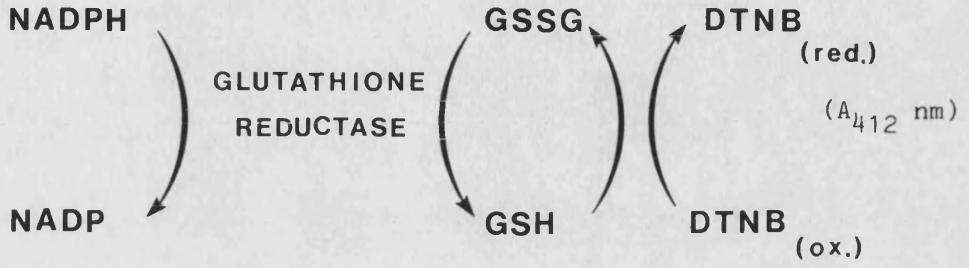
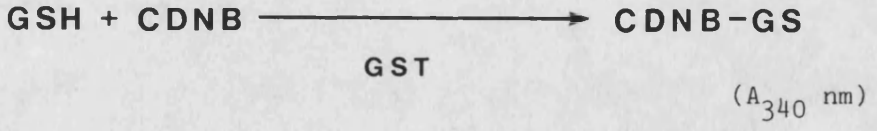


Fig. 4.2 Reaction scheme for glutathione-s-tranferase assay



(ii) GST Assay

GST activity was also measured spectrophotometrically using a modification of the method described by Mozer, Tiemeier and Jaworski (1983).

The rate of GST-mediated conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) was followed as an increase in UV absorption at 340 nm (Fig. 4.2).

Approximately 0.4 g fresh weight of tissue was ground in a glass homogeniser in 20 ml of 0.2 M Tris/HCl buffer, pH 7.8, plus 1 mM EDTA, with 200 mg polyvinylpolypyrrolidone (PVPP), on ice. The extract was strained through four layers of muslin, and spun at 17,000 g for 20 minutes at 4°C, in a Sorvall RC5B centrifuge. The resulting supernatant was decanted and stored on ice as the crude enzyme extract. 250 µl of the enzyme extract was added to 1125 µl 10 mM GSH and 1125 µl 2 mM CDNB. Both reagents were made up in 0.1 M KH_2PO_4 buffer at pH 6.5. The assay was carried out at 25°C, and the rate of $[A_{340}]$nM recorded.

The rate of reaction was read against a GST standard curve, which was produced using equine GST (Sigma). Enzyme activity was expressed as units/g fresh weight of tissue, and as units/mg protein of sample.

(iii) Protein assay

The protein content of the enzyme extracts was determined using the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard.

4.2.3 Replication of Experiments

All data represents the mean results of three samples per treatment carried out on two separate occasions.

4.3 RESULTS

4.3.1 Glutathione

There was a significant increase in the total glutathione content of maize root tissue following applications of dichlormid (Fig. 4.3). At a rate of 1.0% by seed weight, root tissue contained 190% total glutathione compared with untreated tissue. There was a small increase in glutathione content with increasing dichlormid rate from 0.25 to 1.0% by seed weight, however, the greatest response occurred between 0 and 0.25%.

Neither dichormid nor NA had any significant effect upon the total glutathione content of maize shoot tissue at five days after treatment (Figs. 4.3, 4.4). NA also had no effect upon the glutathione content of root tissue (Fig. 4.4).

Fig. 4.3 Effect of dichlormid on glutathione levels in Zea mays var. LG 11 tissue 5 days after treatment

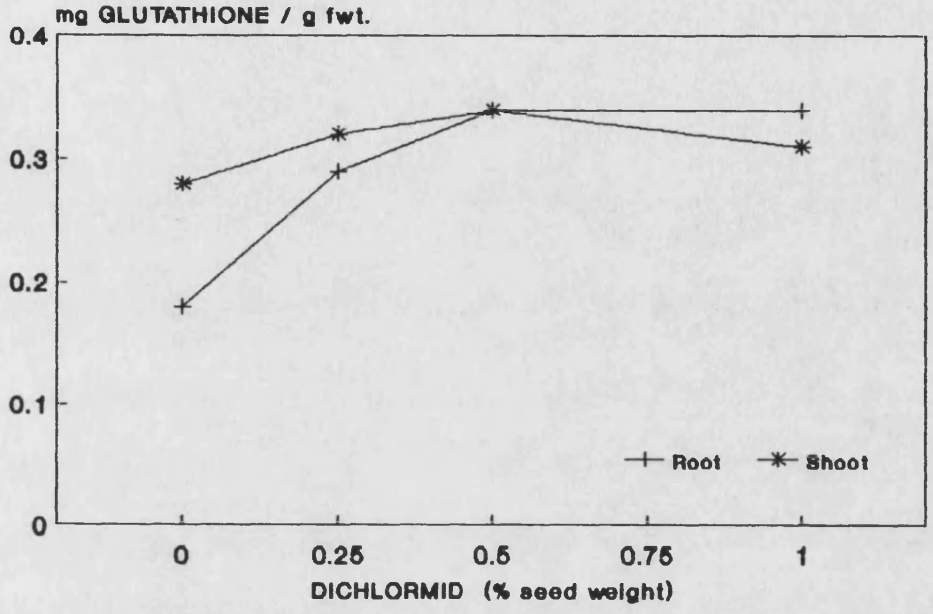
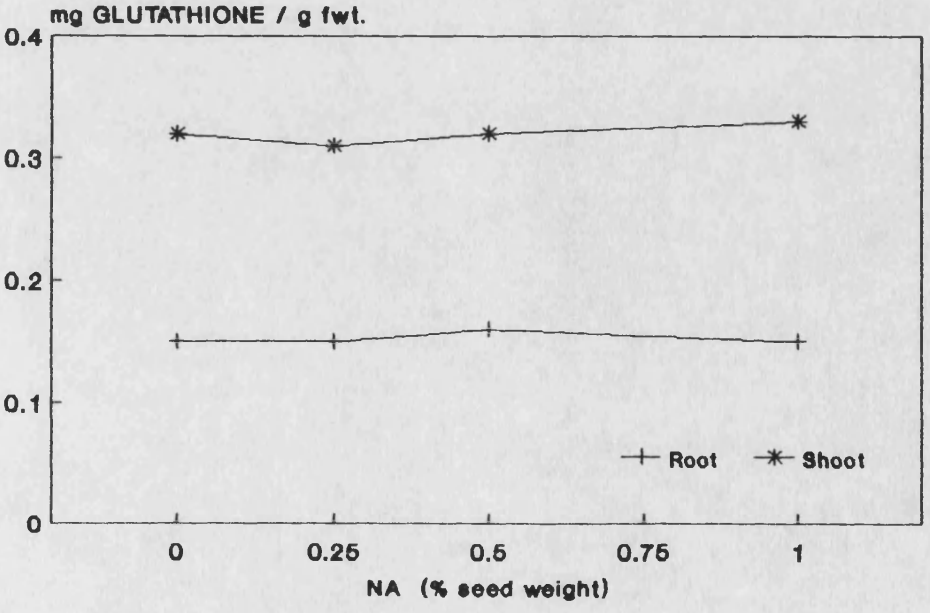


Fig. 4.4 Effect of NA on glutathione levels in Zea mays var. LG 11 tissue 5 days after treatment



4.3.2 GST

Both dichlormid and NA treatments enhanced the activity of GST in shoot tissue (Figs. 4.5–4.8). Significant enhancement of GST activity was apparent when measured either per g fresh weight of plant tissue, or per mg of protein. The response to safener treatment increased with application rate, and GST activity rose to 200–250% of untreated tissue at 1.0% safener by seed weight.

Dichlormid also enhanced GST activity in root tissue. At 1.0% by seed weight, GST activity was 300% and 180% that of untreated root tissue when measured per g fresh weight or per mg protein respectively (Figs. 4.5, 4.6). However, the response to NA was less clear cut. When measured per g fresh weight of tissue, NA enhanced the GST content of root tissue by up to 200% (Fig. 4.7), but when measured per mg protein of tissue, no significant increase was observed (Fig. 4.8). Both NA and dichlormid treatments increased the protein content of root tissue to 175–200% that of untreated tissue, whereas the protein content of shoot tissue did not alter by more than plus or minus 40% of control values (Table 4.1).

Fig. 4.5 Effect of dichlormid on GST activity in Zea mays var. LG 11 tissue 5 days after treatment

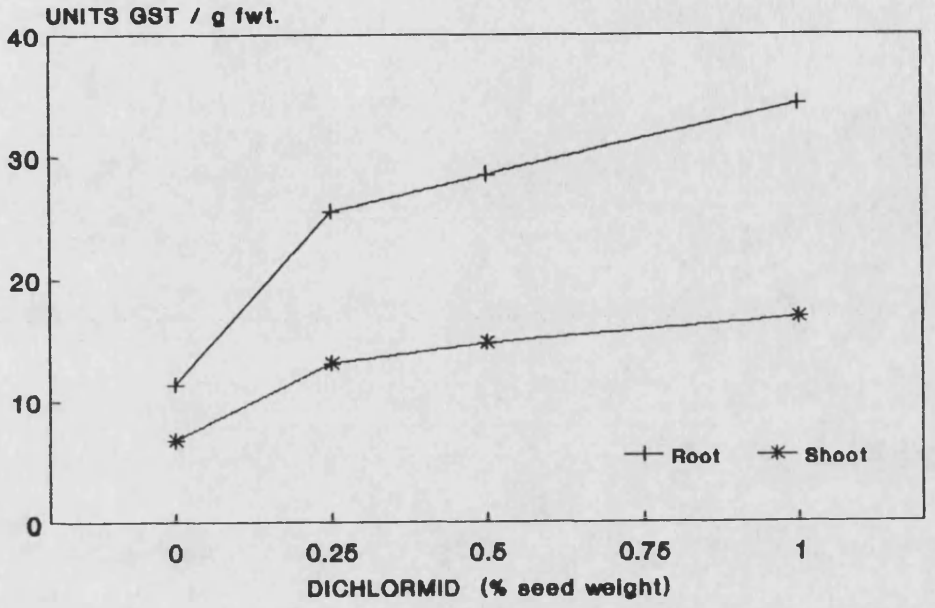


Fig. 4.6 Effect of dichlormid on GST activity/mg protein in Zea mays var. LG 11 tissue 5 days after treatment

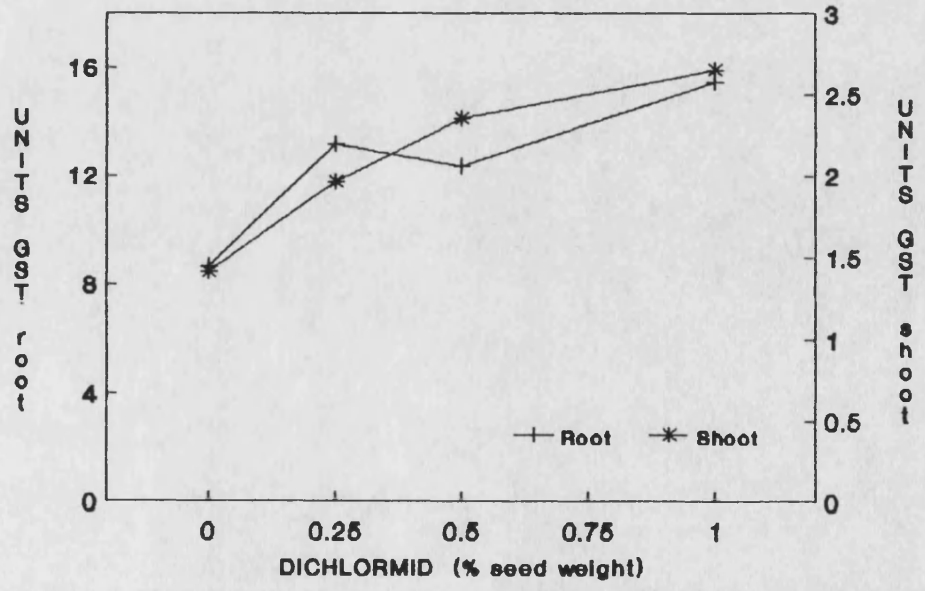


Fig. 4.7 Effect of NA on GST activity in Zea mays var. LG 11 tissue 5 days after treatment

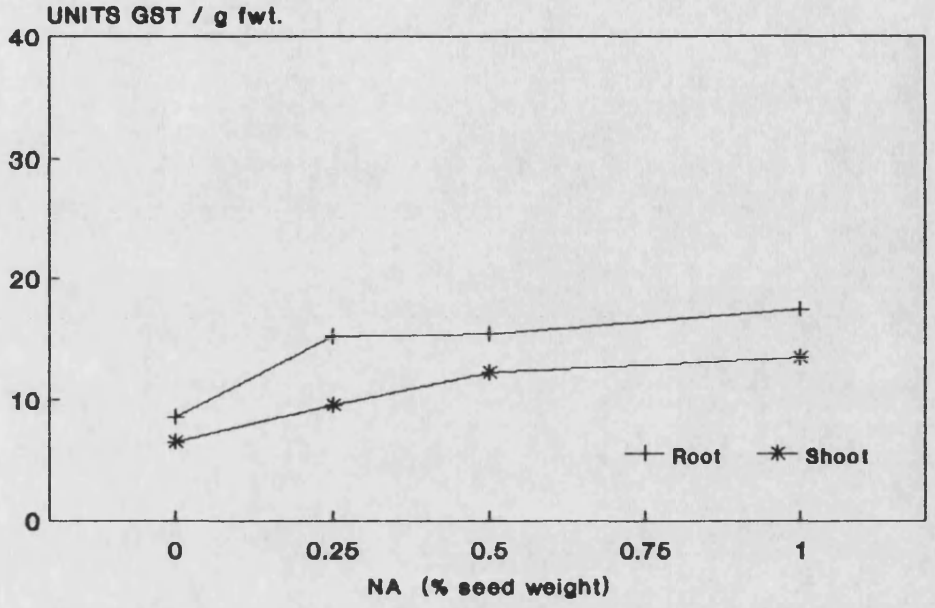


Fig. 4.8 Effect of NA on GST activity /mg protein in Zea mays var. LG 11 tissue 5 days after treatment

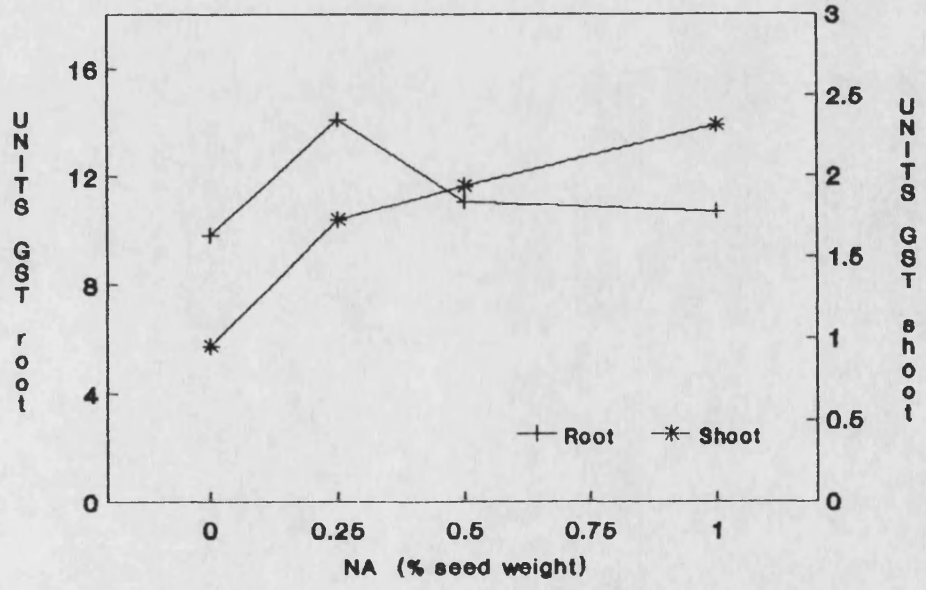


Table 4.1 Effect of safener treatment on protein content of maize plants, 5 days after treatment

Safener	Tissue					
	Root			Shoot		
(% seed wt.)	[1] mgP/g fwt	(S.E.)	% C[2]	mgP/g fwt	(S.E.)	% C
Dichlormid						
0	1.32	(0.17)	100	4.80	(0.05)	100
0.25	1.94	(0.30)	147	6.68	(0.18)	139
0.5	2.32	(0.18)	176	6.29	(0.04)	131
1.0	2.22	(0.22)	168	6.38	(0.38)	133
NA						
0	0.88	(0.11)	100	6.82	(1.60)	100
0.25	1.08	(0.17)	123	5.54	(0.29)	81
0.5	1.40	(0.09)	159	6.33	(0.83)	93
1.0	1.64	(0.14)	186	5.85	(0.14)	71

(1) Values average of 2 x 3 reps. (2) % C = % control value. S.E. = standard error

4.4 DISCUSSION

The response of glutathione (GSH and GSSG) and GST activity in maize to dichlormid treatment, correlated well with other reports in the literature.

The total glutathione (GSH plus GSSG) content of root tissue was elevated from approximately 0.15 mg/g fwt tissue to 0.35 mg/g fwt. Other researchers have found total glutathione levels of betwen 0.1 and 0.15 mg/g fwt root in maize, and an elevation of X2 to X3 with dichlormid treatment (Carringer, Rieck and Bush, 1978; Lay and Casida, 1976, 1978; Lay, Hubbel and Casida, 1975; Lay and Niland, 1985; Stephenson, Ali and Ashton, 1983). As with these results, the response of glutathione levels to dichlormid treatment was found to be concentration dependent.

No significant response of glutathione in shoot tissue was observed with dichlormid in this trial. Most other reports have been limited to glutathione responses in root tissue. However, Lay and Casida (1978) reported an elevation of glutathione in maize shoot tissue of X1.5 with dichlormid application to the roots.

The observed enhancement of GST activity in both shoot and root tissue of X2 to X2.5 with dichlormid treatment also corresponded with other reports (Lay and Casida, 1976, 1978; Mozer, Tiemeier and Jaworski, 1983). However, Lay, Hubbel and Casida (1975) reported elevation of GST activity in maize roots by dichlormid of X7, and

conversely Lay and Niland (1985) found enhancement only in shoot tissue, and not in the root. Again, where GST elevation was observed in response to dichlormid, the effect was concentration dependent as reported here.

NA treatment did not promote glutathione levels in either root or shoot tissue. This was similar to the findings of Kömives et al. (1985b) and Lay and Casida (1976). No report of effects in shoot tissue was given by these workers.

GST activity was enhanced by a factor of X2 in both shoot and root tissue in response to NA, when activity was measured per g fwt material. However, the root response was not apparent when GST activity was measured against the protein content of the tissue. Fedtke (1981), Kömives et al. (1985b) and Mozer, Tiemeier and Jaworski (1983) all reported an enhancement of GST activity in maize tissue following NA treatment by a factor of X1.5 to X2. However, Lay and Casida (1976) found no effect of NA upon GST activity. A possible explanation of these conflicting reports is the choice of substrate used to assay GST activity. CDNB was used in this investigation and also by Fedtke (1981), Kömives et al. (1985b) and Mozer, Tiemeier and Jaworski (1983), whereas EPTC sulfoxide was chosen as the substrate by Lay and Casida (1976). GST has been found to occur as at least two isoenzymes (Guddewar and Dauterman, 1979; Edwards and Owen, 1986b, 1988; Mozer, Tiemeier and Jaworski, 1983), which exhibit different substrate specificities (Edwards and Owen, 1986b). Edwards and Owen (1986b, 1988) also

reported that safener treatment enhanced the activity of only one of the two isoenzymes present. Hence, it may be possible that NA enhances the activity of a GST isoenzyme which is active with CDNB, but not EPTC sulfoxide.

Because of the specificity of GST isoenzymes induced following safener treatment, the use of the general GST substrate CDNB has been questioned as a measure of the ability of a plant to metabolise specific herbicides (Edwards and Owen, 1986a). Gronwald et al. (1987) showed that NA treatment of sorghum resulted in a X2 increase in GST activity when measured with CDNB, but a X17 increase in activity, when ^{14}C metolachlor was used as the substrate. Despite this, NA enhancement of GST activity towards CDNB was observed in this experiment and by other researchers, and indicated a biochemical response in maize to this safener. However, this response did not necessarily indicate an enhanced ability to metabolise specific herbicides, such as the thiocarbamates.

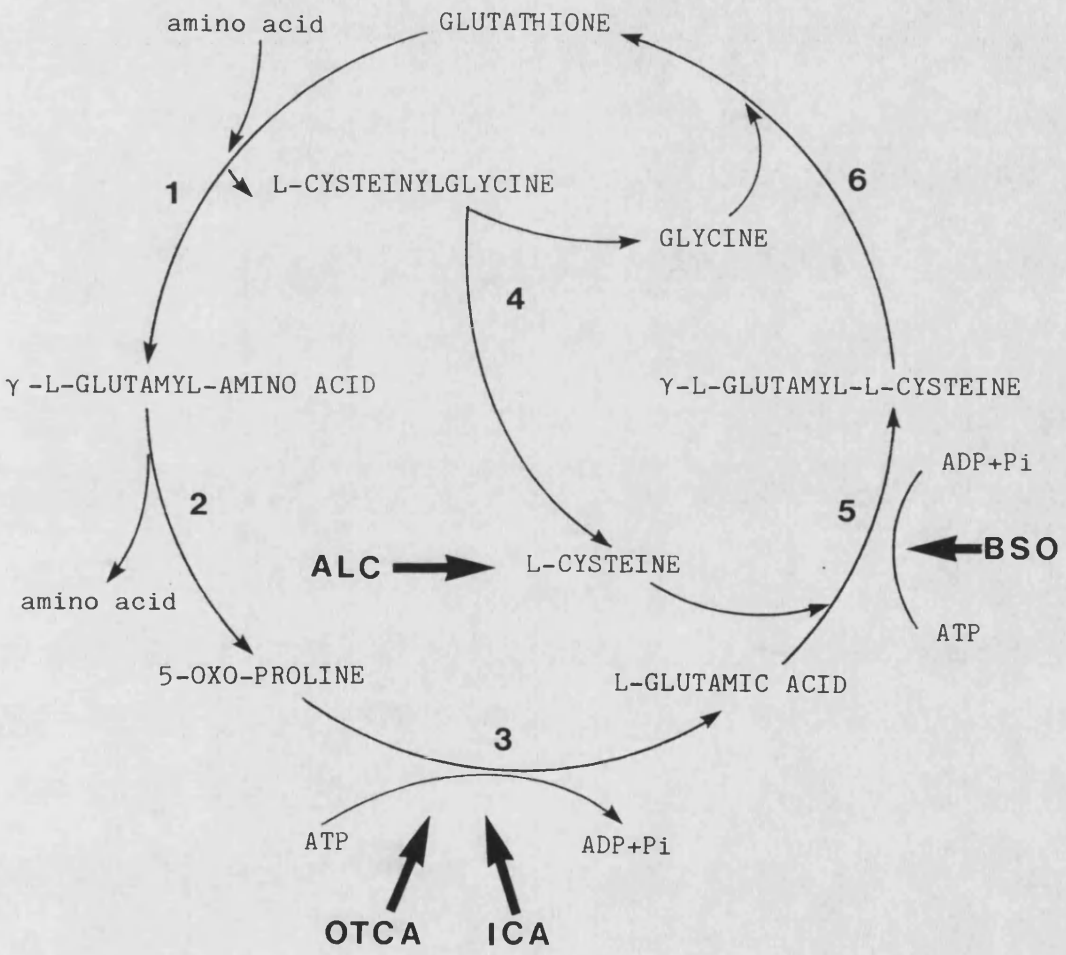
5. POTENTIAL MODULATORS OF GLUTATHIONE IN PLANTS: EFFECTS ON PISUM SATIVUM LEAF TISSUE AND INTERACTION WITH PARAQUAT

5.1 INTRODUCTION

Glutathione (L-γ-glutamyl-L-cysteinylglycine), occurs in plant, animal and bacterial cells (Meister, 1983; Rennenberg, 1982). It functions in the reduction of disulphide linkages of proteins and other molecules, and is essential for the synthesis and degradation of proteins, formation of the deoxyribonucleotide precursors of DNA, and regulation of enzymes. It also plays an important role in protecting cells against reactive oxygen compounds and free radicals, and is a co-enzyme for several reactions. Glutathione conjugates with toxic foreign compounds such as drugs and pesticides, and compounds formed in metabolism, as a first step in their detoxification (Meister and Anderson, 1983). It is also thought to be important as a storage and transport form of reduced sulphur in plants (Rennenberg, 1982).

The synthesis and degradation of glutathione in animal cells is believed to proceed via a series of enzyme catalysed reactions described by the γ-glutamyl cycle (Figure 5.1) (Meister and Anderson, 1983; Rennenberg, 1982). Research into the metabolism of glutathione in animal cells has resulted in the discovery and development of several compounds which modulate glutathione levels (Meister, 1983). These act either as inhibitors of enzymes in the γ-glutamyl cycle, thus lowering glutathione levels, or as

Fig. 5.1 Synthesis and degradation of glutathione in the γ-glutamyl cycle (after Rennenberg, 1982)



ENZYMES.

1. γ-glutamyl transpeptidase
2. γ-glutamyl cyclotransferase
3. 5-oxo-prolinase
4. dipeptidase
5. γ-glutamylcysteine synthetase
6. glutathione synthetase

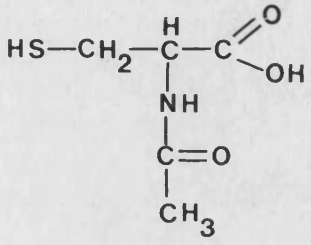
alternative sources of substrates for glutathione synthesis, thus raising glutathione. The sites of action of some of these modulators are also shown in Figure 5.1.

L-buthionine-[S,R]-sulfoximine (BSO) has been found to be a potent and specific inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979). Mice treated with the compound showed a rapid decline in glutathione in kidney, liver, plasma, pancreas and muscle tissues (Meister, 1983). Methionine sufloximine (MSO) and prothionine sulfoximine (PSO) also inhibited γ-glutatmylcysteine synthetase and reduced glutathione levels in animals, but to a lesser extent than BSO (Griffith, Anderson and Meister, 1979; Griffith and Meister, 1978, 1979; Meister, 1983). MSO and its α-alkyl analogs also inhibit glutamine synthetase (Griffith and Meister, 1978). However, BSO and PSO were found to be selective for γ-glutamyl cysteine synthetase, and had no effect on glutamine synthesis (Griffith and Meister, 1979).

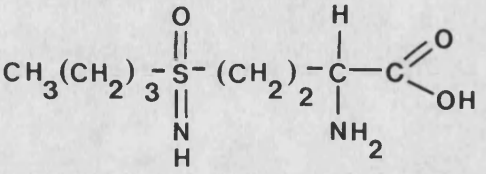
2-imidazolidone-4-carboxylic acid (ICA) administered to mice, inhibited the activity of 5-oxoprolinase, the enzyme that converts 5-oxo-proline to L-glutamic acid. (Van Der Werf *et al.*, 1973; Van Der Werf, Stephani and Meister, 1974). This led to an accumulation of 5-oxo-proline. However, no evidence of its effects upon glutathione levels was presented.

Several compounds have been reported to enhance the glutathione content of treated tissues. N-acetyl-L-cysteine (ALC) and

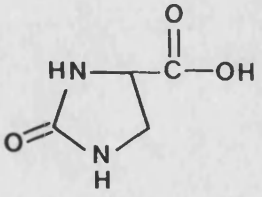
Fig. 5.2 Modulators of glutathione synthesis used in this investigation



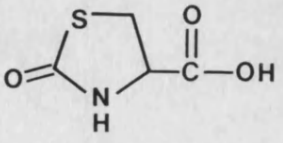
N-ACETYL-L-CYSTEINE



BUTHIONINE-[S,R]-SULFOXIMINE



2-IMIDAZOLIDONE-4-CARBOXYLIC ACID



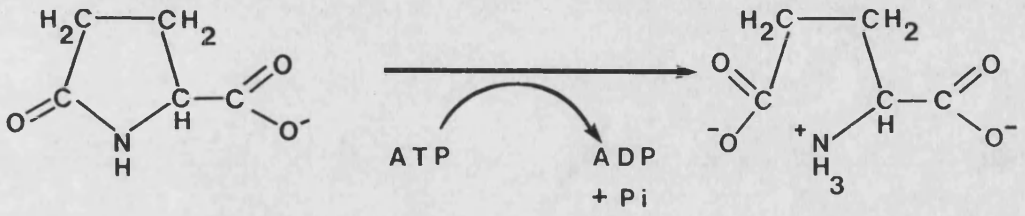
2-OXO-4-THIAZOLIDINE CARBOXYLIC ACID

2-oxo-4-thiazolidine carboxylic acid (OTCA) enhanced glutathione levels in treated mice (Williamson, Boettcher and Meister, 1982; Williamson and Meister, 1981), with OTCA being more effective than ALC (Meister, 1983). OTCA was found to be converted by 5-oxo-prolinase to L-cysteine (Williamson, Boettcher and Meister, 1982; Williamson and Meister, 1981) (Figure 5.3). The enhanced level of L-cysteine led to an increase in the production of glutathione via γ-glutamylcysteine synthetase and glutathione synthetase (Fig. 5.1). The enhancement of glutathione by ALC was believed to be due to deacylation of the compound in vivo to release cysteine (Williamson, Boettcher and Meister, 1982).

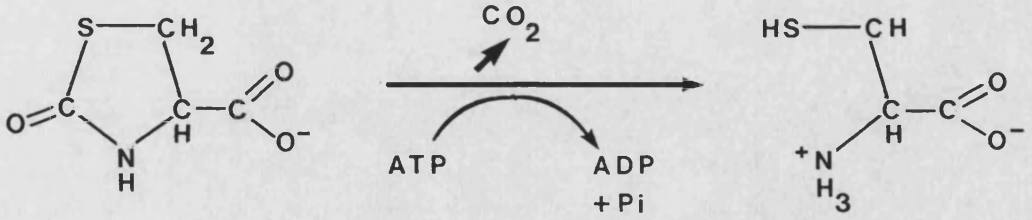
The synthesis of glutathione in plants is believed to proceed via the same series of enzyme catalysed reactions as observed in the γ-glutamyl cycle in animal cells. However, the pathway of glutathione degradation in plants is not yet certain (Rennenberg, 1982). Both γ-glutamylcyclotransferase and 5-oxo-prolinase activity have been reported to occur in plants, but the participation of γ-glutamyltranspeptidase in glutathione degradation is uncertain.

Because of the similarity of glutathione metabolism in plants and animal cells, these compounds may also prove to be effective in modulating glutathione levels in plants. MSO has been reported to lower the glutathione levels of tobacco cells in suspension culture (Rennenberg and Uthemann, 1980). OTCA and L-thiazolidine-4-carboxylic acid (TCA) were found to increase the thiol content of excised maize roots (Hilton and Pillai, 1986, 1987). Meister (1983)

Fig. 5.3 Reactions catalysed by 5-oxo-prolinase (after Meister, 1981)



5-OXOPROLINE GLUTAMATE



2-OXO-4-THIAZOLIDINE CARBOXYLIC ACID L-CYSTEINE

suggested that enhancers of glutathione such as OTCA might prove effective as safeners against herbicides metabolised via glutathione conjugation. Hilton and Pillai (1986, 1987) reported some success in overcoming tridiphane toxicity in maize with OTCA, and tridiphane damage to maize and sorghum, and alachlor to sorghum with TCA.

The aim of this section of work was to investigate the effect of four potential modulators of glutathione synthesis in plant leaf tissue. BSO and ICA were chosen as potential inhibitors of glutathione synthesis, and ALC and OTCA as potential enhancers. Leaf tissue of pea (Pisum sativum) was chosen as a convenient plant material for treatment.

Glutathione plays an important role in the protection of plant chloroplasts against damage by superoxide and hydrogen peroxide, which are generated during photosynthesis under normal conditions (Dodge, 1989; Figure 5.4). The bipyridyl herbicides paraquat and diquat enhance the production of superoxide by diverting electron flow from ferredoxin in photosystem I to oxygen, which leads to the production of hydrogen peroxide and free radicals such as hydroxyl ($OH^{\cdot -}$) (Dodge, 1983b). Modulation of glutathione levels in plants might result in a modification of paraquat toxicity. To this end, the effect of some of the glutathione modulators on paraquat damage to leaf material was examined.

Fig. 5.4 Chloroplast scavanging enzymes for superoxide and hydrogen peroxide showing 'ascorbate-glutathione' cycle and action of paraquat (after Dodge, 1989)

PQ^{2+} paraquat

$PQ^{\cdot -}$ reduced paraquat

$O_2^{\cdot -}$ superoxide

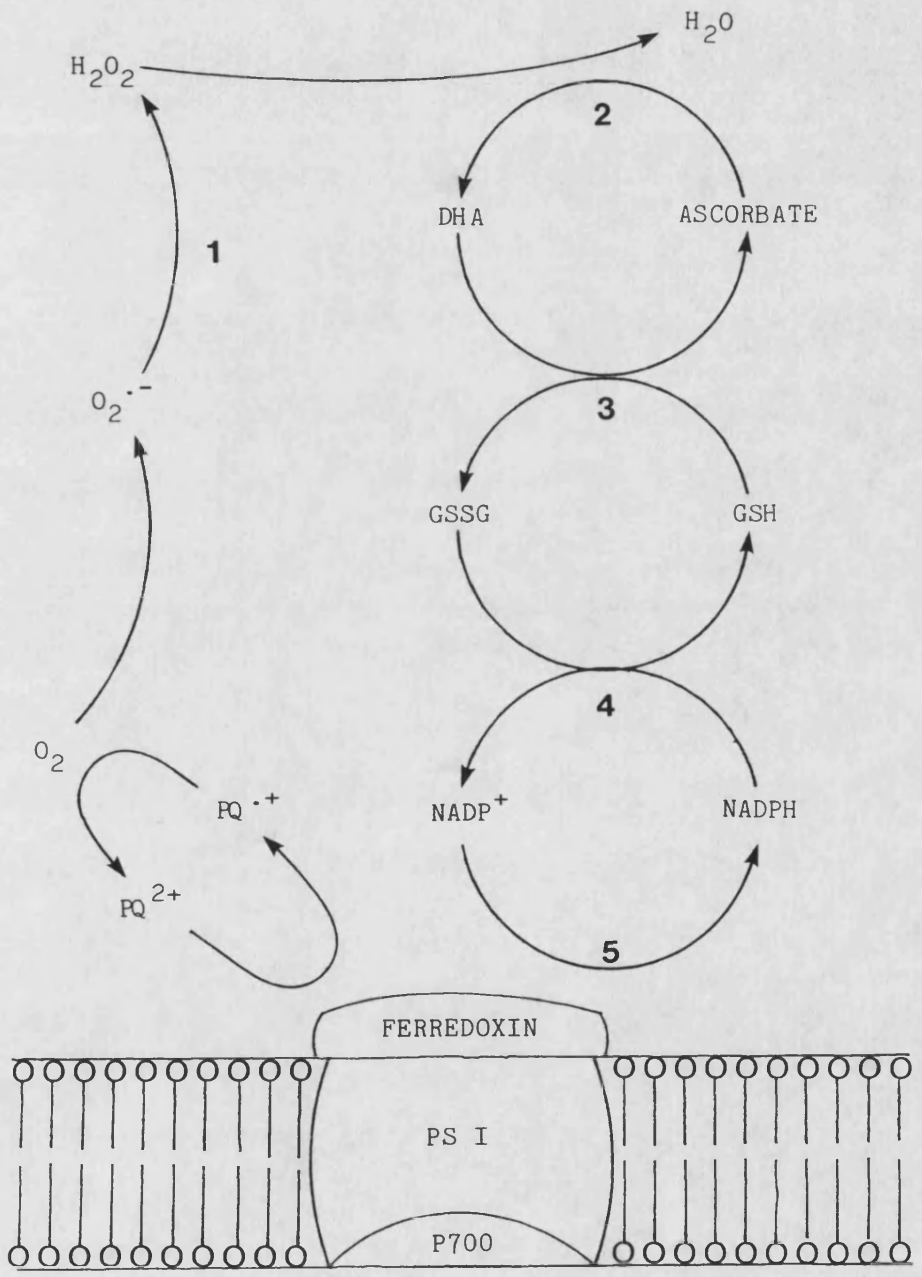
DHA dehydroascorbate

GSH reduced glutathione

GSSG oxidised glutathione

PS I photosystem I

1. superoxide dismutase
2. ascorbate peroxidase
3. dehydroascorbate reductase
4. glutathione reductase
5. ferredoxin-$NADPH^+$ reductase


H_2O
H_2O_2
2
DHA
ASCORBATE
1
$O_2^{\cdot -}$
3
GSSG
GSH
4
O_2
$PQ^{\cdot +}$
$NADP^+$
NADPH
PQ^{2+}
5
FERREDOXIN
PS I
P700

5.2 MATERIALS AND METHODS

5.2.1 Plant Material and Test Conditions

Pea (Pisum sativum var. Meteor) plants were grown in trays in Levington Universal Compost in a glasshouse under natural daylight conditions, or natural daylight extended to a 14 hr photoperiod (Thorne 400 W mercury vapour lamps). The mean air temperature during growth was 22°C. Leaf discs (10 mm diameter) were cut from the youngest fully expanded leaves on plants 14-21 days after sowing (when three sets of leaves had developed). Three discs were floated on test solutions in 5 cm diameter petri dishes and placed in a cabinet under continuous fluorescent light (390 $\mu Em^{-2}s^{-1}$) at a temperature of 27°C. Treatment periods were between 0 and 72 hours.

5.2.2 Chemicals

L-buthionine-[S,R] -sulfoximine (BSO), 2-imidazolidone-4-carboxylic acid (ICA), 2-oxo-4-thiazolidine-carboxylic acid (OTCA), and N-acetyl-L-cysteine (ALC) were obtained from Sigma.

All test chemicals and the herbicide paraquat (technical grade) were dissolved directly in distilled water, and made up to the appropriate test concentrations. BSO, ICA, OTCA and ALC were used at 10^{-4} M to 10^{-7} M for initial glutathione modulation experiments. For the investigation of a possible interaction with paraquat, test chemicals were applied at 10^{-4} M and paraquat at 10^{-7} M.

5.2.3 Glutathione assay

The glutathione content of all three leaf discs per test solution was assayed according to the method described in Section 4.2.2 of this thesis.

5.2.4 Chlorophyll and Carotenoid Determinations

The chlorophyll and carotenoid content of paraquat treated leaf discs was determined according to the method of Lichtenthaler and Wellburn (1983).

Leaf discs were soaked in 95% v/v ethanol for one to two days in the dark. Pigments were estimated from the absorbance of the ethanol extract at 470, 649 and 665 nm using a Shimadzu UV-260 UV-VIS recording spectrophotometer, according to the equations;

$$\text{Chlorophyll a } (\mu g/ml) = 13.95\ (A_{665}) - 6.88\ (A_{649})$$

$$\text{Chlorophyll b } (\mu g/ml) = 24.96\ (A_{649}) - 7.32\ (A_{665})$$

$$\text{Carotenoids } (\mu g/ml) = \frac{1000\ (A_{470}) - 2.05\ Ca - 114.8\ Cb}{245}$$

where Ca = Chlorophyll a

Cb = Chlorophyll b

5.2.5 Replication of Experiments

All results represent the mean of three measurements repeated on two separate occasions.

5.3 RESULTS

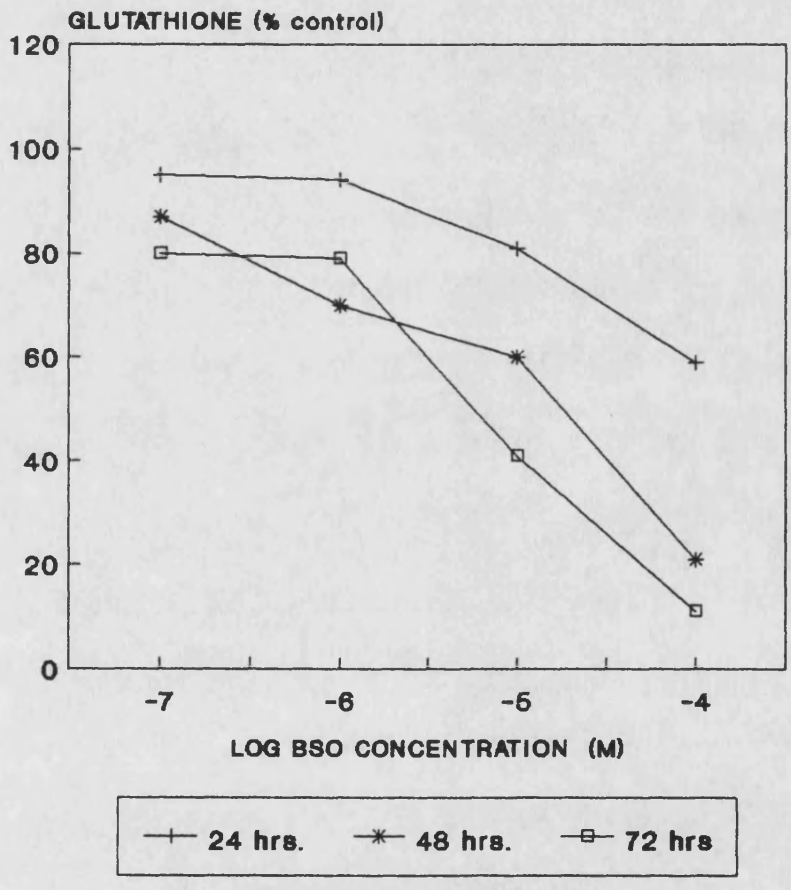
5.3.1 Experiment 1. Glutathione Modulation

All BSO treatments led to a decrease in glutathione content of pea leaf discs (Fig. 5.5). The reduction in glutathione content was both concentration and time dependent. At 10^{-4} M BSO, glutathione fell to 59% of untreated control at 24 hours, and 11% of control after 72 hours. No visual symptoms such as pigment bleaching were associated with the BSO effect on glutathione.

ALC and OTCA both enhanced the glutathione content of treated discs compared with controls (Figs. 5.6, 5.7). At 24 hrs 10^{-4} M OTCA enhanced glutathione to 164% of control, and this value fell to 130% by 72 hours. All other OTCA concentrations failed to enhance glutathione, with the exception of 10^{-5} M at 24 hours. 10^{-4} M ALC enhanced glutathione at 24 and 48 hours to 167% and 134% of controls respectively. The response to ALC did not appear to be concentration dependent, but did show an increase with time, with the exception of 10^{-4} M.

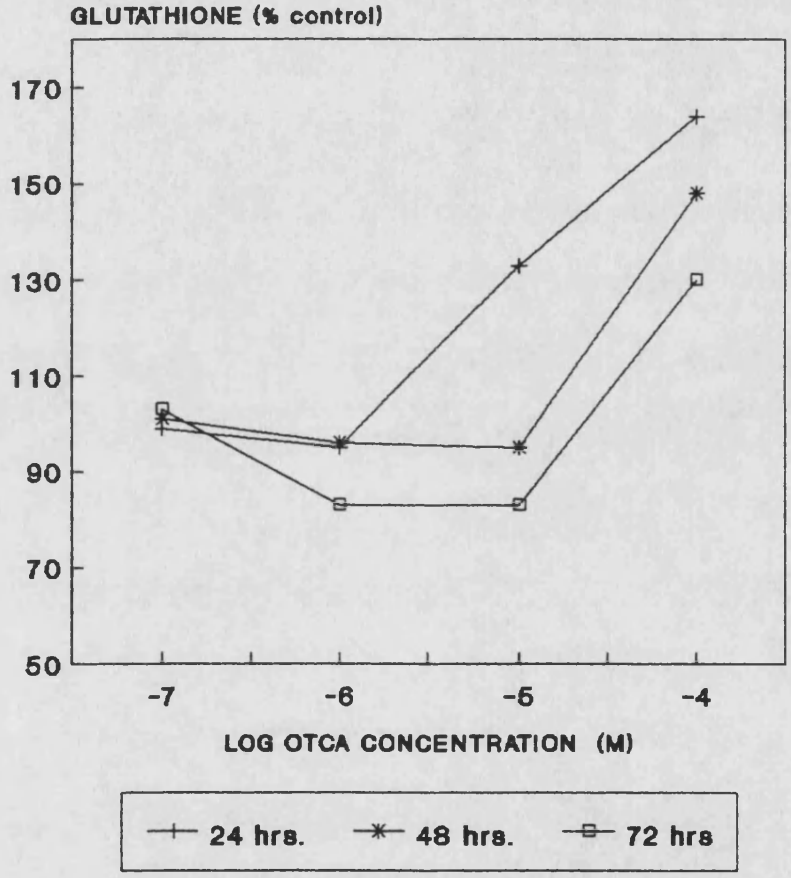
ICA failed to reduce the glutathione content of pea leaf tissue in

Fig. 5.5
BSO modulation of glutathione in pea leaf tissue



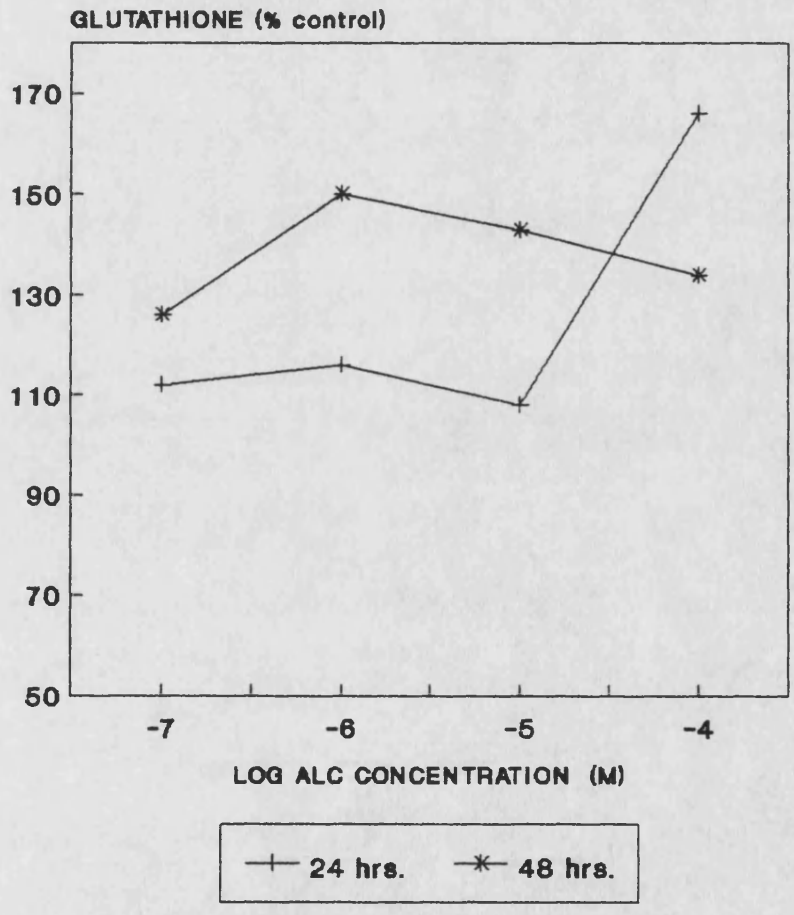
Control values (mg/g fwt.)
24 hr 0.329 +/-0.04 48 hr 0.399 +/-0.05
72 hr 0.611 +/-0.11

Fig. 5.6
OTCA modulation of glutathione in pea leaf tissue



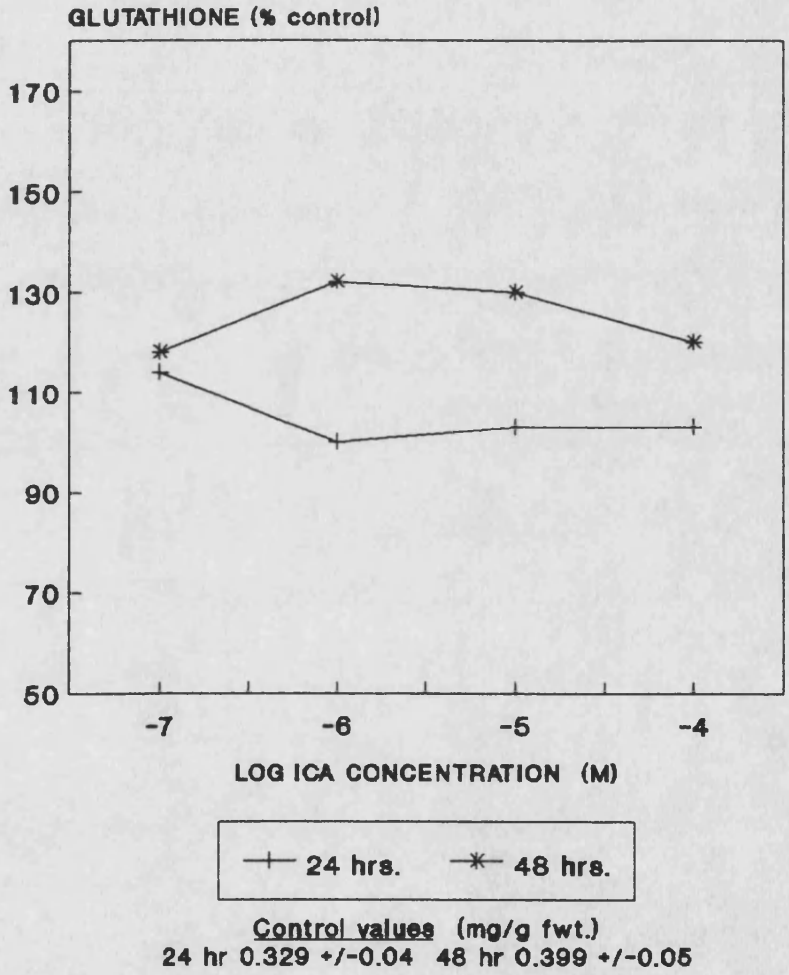
Control values (mg/g fwt.)
24 hr 0.329 +/-0.04 48 hr 0.399 +/-0.05
72 hr 0.611 +/-0.11

Fig. 5.7
ALC modulation of glutathione in pea leaf tissue



Control values (mg/g fwt.)
24 hr 0.329 +/-0.04 48 hr 0.399 +/-0.05

Fig. 5.8
ICA modulation of glutathione in pea leaf tissue



Control values (mg/g fwt.)
24 hr 0.329 +/-0.04 48 hr 0.399 +/-0.05

this experiment (Fig. 5.8). At 24 hrs, treatment values were similar to controls, whilst by 48 hrs glutathione values were 120-130% of controls. The enhancement of glutathione was not concentration dependent.

No visual effects of ICA, ALC or OTCA on leaf disc pigmentation were observed.

5.3.2 Experiment 2. Effect of Glutathione Modulators on Paraquat Damage to Pea Leaf Tissue

Following on from the results of glutathione modulation in Experiment 1, BSO, OTCA and ALC were investigated as potential synergists or antagonists of the bleaching effect of paraquat on leaf tissue.

Initial trials indicated that 10^{-7} M paraquat caused a 10-20% bleaching of leaf discs over a 48-72 hr period. Paraquat free treatments caused no loss of pigment. It was hoped that by using paraquat at this concentration a partial bleaching of discs could be produced, which would allow both antagonistic and synergistic effects of glutathione modulators upon paraquat damage to be observed. BSO, OTCA and ALC were applied at 10^{-4} M, as at this concentration they produced the most conclusive effects upon glutathione levels in the leaf discs in Experiment 1.

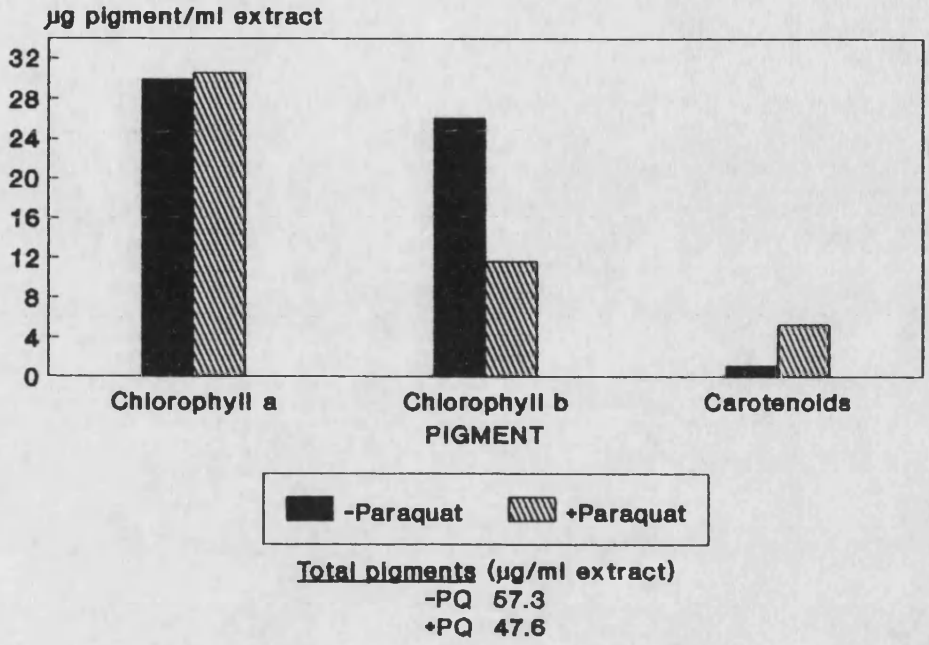
Figures 5.9-5.12 show the effect of 10^{-7} M paraquat for 48 hrs on leaf pigment levels with 24 hrs pretreatment with H_2O or glutathione modulators. Values represent pigment concentrations in µg/ml ethanol for three leaf discs in 5 ml solvent (mean fresh weight of three leaf discs was 0.229 ± 0.002 g).

10^{-7} M paraquat had no effect upon chlorophyll a content of discs, but caused a reduction in chlorophyll b of 40-50%, and an increase in total carotenoids of X2-X3. Total pigment concentrations fell to approximately 85% of control. None of the glutathione modulators had any significant effect upon paraquat damage as measured by pigment concentrations, or by visual symptoms.

A repeat of the experiment was carried out, but with simultaneous paraquat and modulator treatment and over 96 hrs rather than 72 hrs. Paraquat treatment had less effect on pigment levels compared with controls than in the previous experiment (Figs. 5.13-5.16). Chlorophyll b was 70-80% of control levels, and total pigments 92-96% of control. This change in response was accounted for by a slight degeneration of control discs by 96 hrs, with consequent damage to pigments. Glutathione modulators again had no significant effect upon paraquat damage.

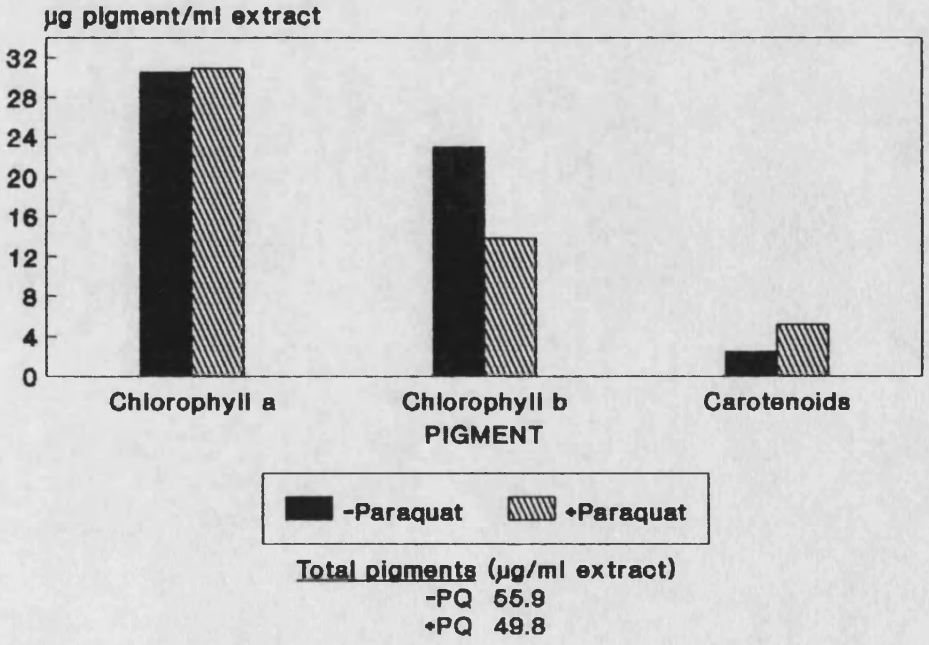
None of the glutathione modulators had any significant effect upon pigment concentrations in the absence of paraquat.

Fig. 5.9 Effect of paraquat on pea leaf pigment levels + 48 hrs treatment
a) Water pretreatment (24 hrs)



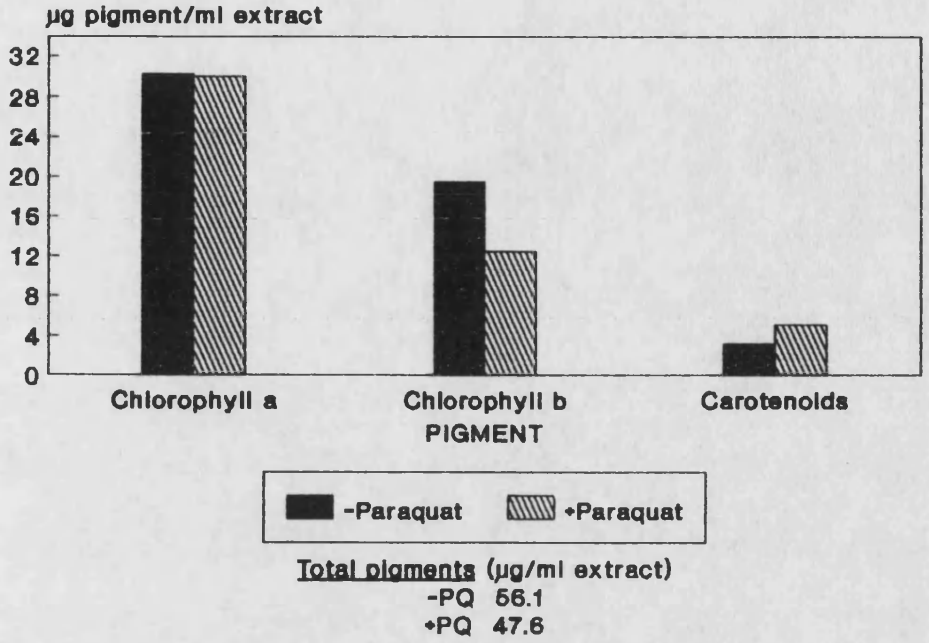
Total pigments (µg/ml extract)
-PQ 57.3
+PQ 47.6

Fig. 5.10 Effect of paraquat on pea leaf pigment levels + 48 hrs treatment
b) OTCA pretreatment (24 hrs)



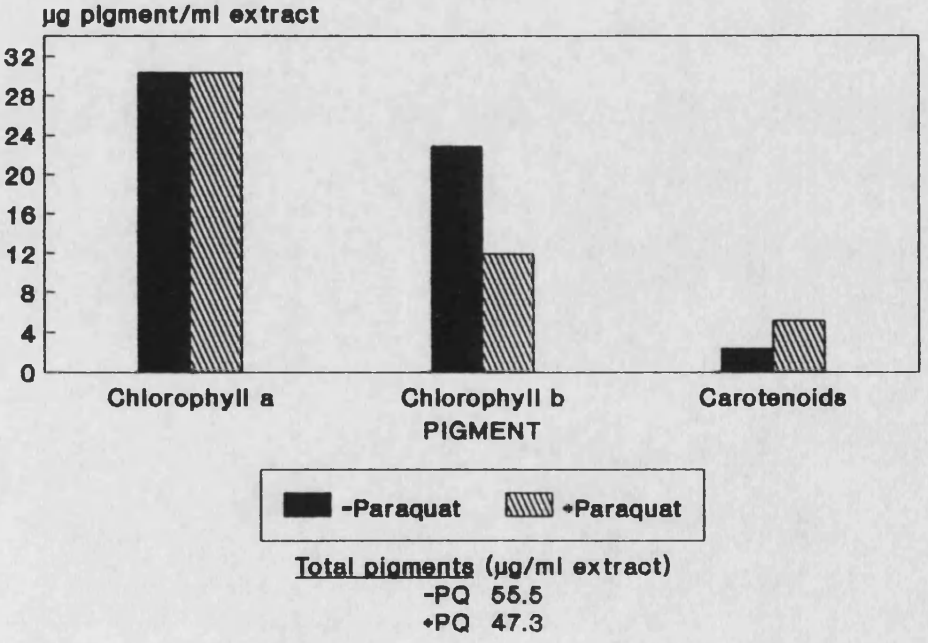
Total pigments (µg/ml extract)
-PQ 55.9
+PQ 49.8

Fig. 5.11 Effect of paraquat on pea leaf pigment levels + 48 hrs treatment c) BSO pretreatment (24 hrs)



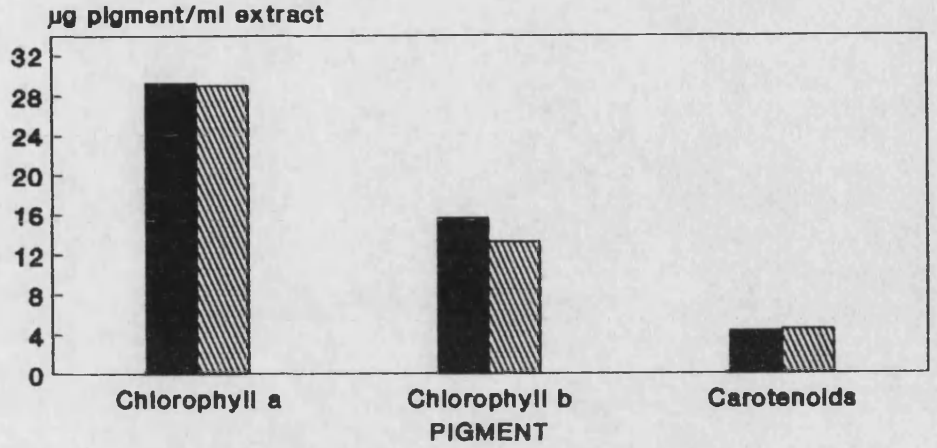
Total pigments (µg/ml extract)
-PQ 56.1
+PQ 47.6

Fig. 5.12 Effect of paraquat on pea leaf pigment levels + 48 hrs treatment d) ALC pretreatment (24 hrs)



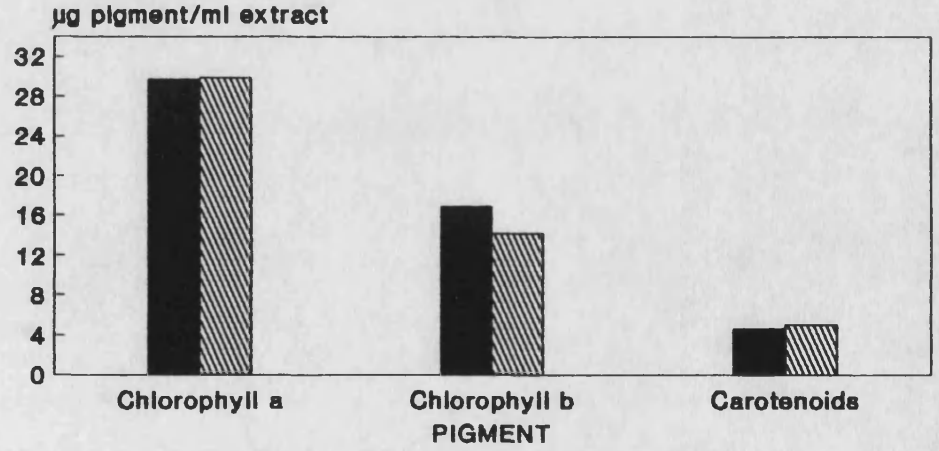
Total pigments (µg/ml extract)
-PQ 55.5
+PQ 47.3

Fig. 5.13 Effect of paraquat on pea leaf pigment levels + 96 hrs treatment
a) Water (simultaneous treatment)



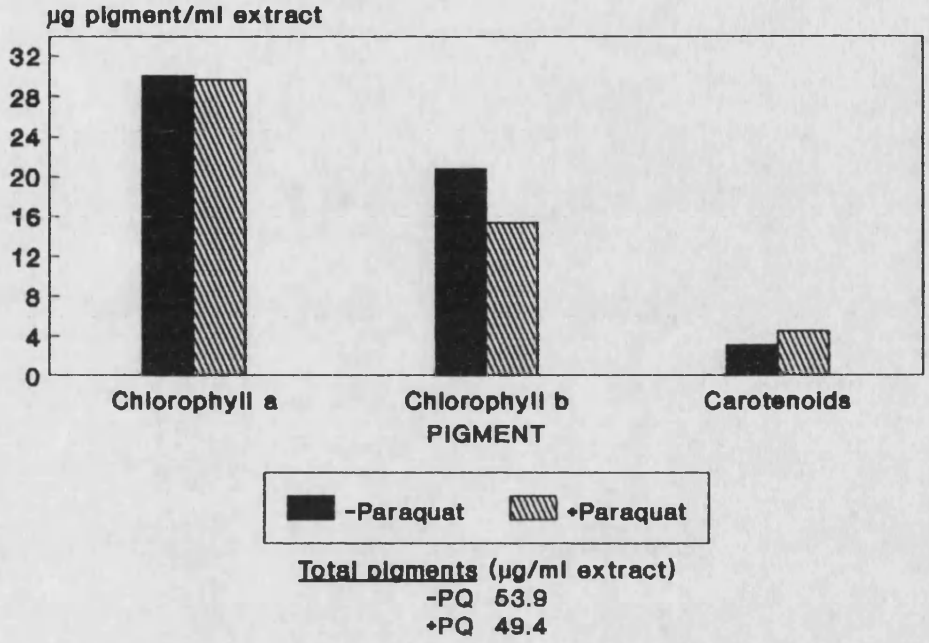
Total pigments (µg/ml extract)
-PQ 49.1
+PQ 46.7

Fig. 5.14 Effect of paraquat on pea leaf pigment levels + 96 hrs treatment
b) OTCA (simultaneous treatment)



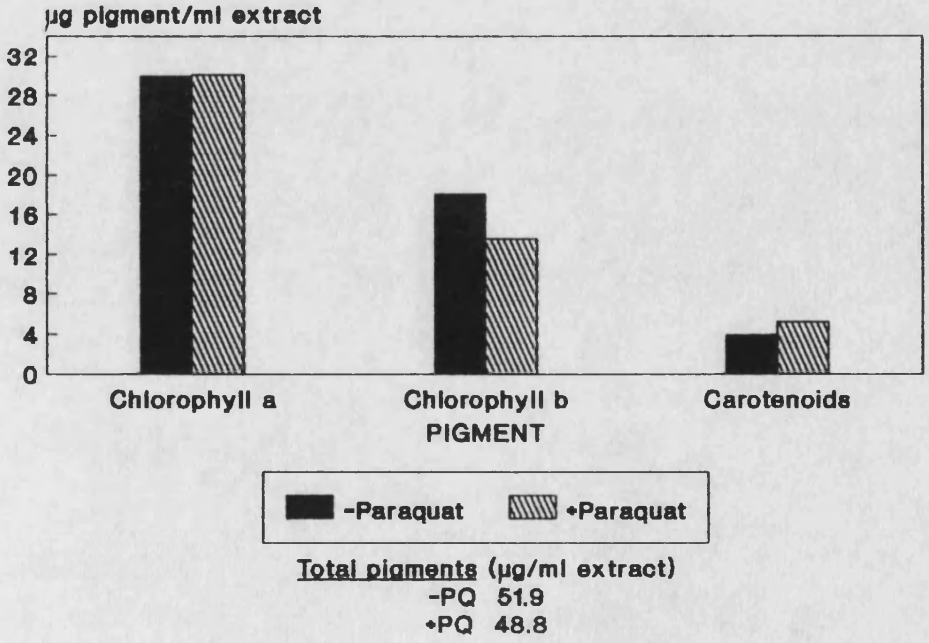
Total pigments (µg/ml extract)
-PQ 51.2
+PQ 49.2

Fig. 5.15 Effect of paraquat on pea leaf pigment levels + 96 hrs treatment c) BSO (simultaneous treatment)



Total pigments (µg/ml extract)
-PQ 53.9
+PQ 49.4

Fig. 5.16 Effect of paraquat on pea leaf pigment levels + 96 hrs treatment d) ALC (simultaneous treatment)



Total pigments (µg/ml extract)
-PQ 51.9
+PQ 48.8

5.4 DISCUSSION

Buthionine sulfoximine (BSO) was found to be effective in reducing the glutathione content of leaf tissue. Treatment at 10^{-4} M for three days resulted in glutathione levels of approximately 10% of untreated tissue. The response was dependent upon both the dose and length of time of treatment. This result was consistent with the action of a competitive inhibitor of an enzyme involved in the biosynthesis of glutathione. Hence, BSO appeared to be a potent inhibitor of γ-glutamylcysteine synthetase in plant tissue, as had been previously reported with animal tissue (Griffith and Meister, 1979; Meister, 1983). No previous reports of the effect of BSO in plant tissue have been made. However, Rennenberg and Uthemann (1980) found that methionine sulfoximine (MSO), another inhibitor of γ-glutamylcysteine synthetase, reduced glutathione levels in suspension cultured tobacco cells.

The decline in glutathione levels to 10% of untreated tissue within 72 hours of inhibition of biosynthes is also suggested a rapid turnover of glutathione in leaf tissue under normal conditions.

2-oxothiazolidine-4-carboxylic acid (OTCA) enhanced glutathione concentration in leaf tissue to 130-160% of control at 10^{-4} M, depending upon the length of treatment. Hilton and Pillai (1986) found a similar response in intact maize root tissue exposed to this concentration of OTCA, with glutathione levels dropping from 200 to 100% of control over 1 to 3 days treatment. Excised maize

roots incubated in 10^{-4} M OTCA for 0.5 to 4 hrs had glutathione concentrations of 120-130% of controls. These results suggested that OTCA may be acting as a source of cystine in plant tissue due to enzymatic conversion by 5-oxo-prolinase, as has been reported in animal tissue (Williamson, Boettcher and Meister, 1982; Williamson and Meister, 1981). The gradual decline in the elevated glutathione levels with time, as observed in this trial and by Hilton and Pillai (1986), would be consistent with OTCA acting as an additional source of cysteine for glutathione synthesis. However, this response might also have been due to increased feedback inhibition of γ-glutamylcysteine synthetase by enhanced glutathione levels, after initial exposure to OTCA (Rennenberg, 1982).

N-acetyl-L-cysteine (ALC) gave some enhancement of glutathione after 48 hours treatment. However, the response was not concentration dependent. No previous reports of ALC effects on glutathione in plant tissue have been made. Williamson, Boettcher and Meister (1982) reported an effect in mouse tissue, and as with these results, OTCA was found to be more effective than ALC. As the enhancement of glutathione by ALC was believed to be due to deacylation of the compound in vivo to release cysteine, (Williamson, Boettcher and Meister, 1982) this might suggest the existence of some N-acylase activity in leaf tissue. However, other mechanisms might also operate.

Although 2-imidazolidone-4-carboxylic (ICA) acid was reported to inhibit 5-oxo-prolinase activity in animal tissue (Van Der Werf

et al., 1973; Van Der Werf, Stephani and Meister, 1974) and hence, might have inhibited glutathione biosynthesis, It did not reduce glutathione levels in leaf tissue in these experiments. A 120-130% enhancement in glutathione was observed after 48 hours treatment. This might suggest that ICA had no effect on 5-oxo-prolinase in plant tissue. However, ALC might have produced a partial inhibition of 5-oxo-prolinase activity resulting in a transient drop in glutathione levels. This in turn could have released feedback inhibition of glutathione on γ-glutamylcysteine synthetase, which might account for the increase in glutathione seen at 48 hours. However, no initial drop in glutathione was observed at 24 hours.

No synergism or antagonism of paraquat damage to leaf tissue was observed with either BSO, OTCA or ALC, despite the previously recorded effects upon leaf glutathione levels. Several possible explanations could account for the lack of modulation of paraquat toxicity by these compounds. Changes in cytosolic glutathione were measured in this experiment, not the glutathione content of chloroplasts. Thus, the observed effects of the modulators upon glutathione levels may not have be representative of changes occurring in the chloroplast, the site of paraquat action. Alternatively, the response to glutathione modulators may have changed in the presence of paraquat. No measurement of glutathione levels was made with paraquat treatments. However, Hilton and Pillai (1987) observed that thiol changes measured in maize with OTCA and TCA treatments did not occur in the presence of alachlor. Herbicides have been reported to enhance the glutathione content of

various plant tissues (Hilton and Pillai, 1986; Stephenson, Ali and Ashton, 1983). It is possible that the low paraquat stress used in these treatments may have induced glutathione levels in leaf tissue, counteracting the effect of BSO. Gillham (1986) demonstrated that 10^{-7} M paraquat enhanced glutathione reductase activity in pea leaf tissue by 25%.

Other modulators of glutathione metabolism in animal tissue, besides those investigated here, have been reported (Bridges, Griffith and Meister, 1980; Griffith, Anderson and Meister, 1979; Griffith and Meister, 1978; Meister, 1983; Van Der Werf, Stephani and Meister, 1974; Williamson and Meister, 1982). Investigation of the effect of these in plant tissue, and interaction with herbicides metabolised via glutathione conjugation, and herbicide safeners, could prove valuable. Although not practicable as herbicide safeners or synergists, these compounds might act as useful tools for investigating further the role of glutathione in herbicide metabolism, and the importance of glutathione enhancement in safener action.

6. MODULATION OF GLUTATHIONE IN ZEA MAYS L. WITH BUTHIONINE SULFOXIMINE: INTERACTION WITH EPTC AND DICHLORMID

6.1 INTRODUCTION

To continue the experiments reported in section five of this thesis, the effect of BSO on glutathione levels in maize was investigated. Previous results had indicated that BSO was effective in reducing glutathione concentrations in pea leaf tissue, when applied directly to the leaf surface. In this present series of experiments, BSO was applied in solution to the plant roots, and the effects upon both root and shoot glutathione were monitored.

The interaction of BSO with dichlormid was also investigated. Dichlormid had proved to be effective in enhancing the glutathione content of maize root tissue (Section 4.3). If BSO was effective in reducing glutathione levels in maize, then results of any interaction with dichlormid might give clues as to the mechanism of dichlormid enhancement of glutathione, i.e. any effect on glutathione biosynthesis before or after γ-glutamylcysteine synthetase in the γ-glutamyl cycle.

As EPTC is metabolised in maize via conjugation with glutathione (Hatzios and Penner, 1982), BSO might be effective in synergising EPTC toxicity. Hilton and Pillai (1986, 1987) reported success in reducing tridiphane and alachlor injury in maize by enhancing glutathione levels with OTCA and TCA treatment. However, no reports

of synergism of EPTC toxicity in maize by altering glutathione levels have been made. Again, possible interaction between BSO and EPTC might give clues as to the importance of glutathione levels in protecting plant tissue against thiocarbamate damage. This also might indicate if the elevation of glutathione as opposed to glutathione-s-transferase activity is a critical factor in the protective action of safeners against this type of herbicide.

6.2 MATERIALS AND METHODS

6.2.1 Plant Material and Growth Conditions

6.2.1.1 Glutathione Modulation Experiments

Maize (Zea mays L. var. LG11) seeds were germinated between moist paper towels for 72 hours in a growth room maintained at 22°C in continuous darkness. Evenly sized seedlings were selected and grown hydroponically in 50% Hoaglands solution (Hoagland and Arnon, 1950), with four plants per 400 ml beaker, in a cabinet, under 16 hours fluorescent light (390 $\mu E\ m^{-2}s^{-1}$), day/night temperatures of 27/19°C respectively. BSO and dichlormid (20% ai) treatments were applied in fresh nutrient solution, after two days growth in the light cabinet, when plants had reached the two leaf growth stage.

6.2.1.2 Interaction of BSO with EPTC - effects on Plant Growth

Three maize (var. LG11) seeds were planted per 9 cm diameter pot in

vermiculite, at a depth of 0.5 cm. Pots were watered with 80 ml of distilled water or 10^{-4} M BSO and placed in a growth room in the dark at 22°C for 48 hours. Pots were then watered with a further 80 ml H_2O ± EPTC/BSO and retained in the growth room for a further 24 hours. The plants were then placed in a cabinet, under 16 hours fluorescent light (390 $\mu E\ m^{-2}s^{-1}$), 27/19°C and left to grow for a further 6 days. All pots were watered at two day intervals with 50 ml 50% Hoaglands solution.

6.2.2 Glutathione Assay

The glutathione content of shoot and root tissue of hydroponically grown maize plants was assayed over a period of 0-72 hours after BSO/dichlormid treatment, according to the procedure described in section 4.2.2. Glutathione content was expressed as mg/g fresh weight of tissue.

6.2.3 Growth Measurements

Three measurements were made to assess the effect of EPTC BSO on maize growth, 9 days after sowing. The length of the second leaf per plant above the soil surface (as in Fig. 3.2) and shoot fresh and dry weights per pot. For dry weights, plant material was dried at 80°C for 48 hours before weighing.

6.2.4 Replication of Experiments

All glutathione data represented the average values of three assay replicates, repeated on two separate occasions.

Two replicates per treatment were used in the growth trial. Leaf length values represented the average of all six plants in the two replicates. Fresh and dry weight values were the average of the two replicates.

6.3 RESULTS

Buthionine sulfoximine (BSO) was found to be an effective modulator of glutathione in maize. Applications of 10^{-6} M and above to the roots of two leaf plants, resulted in a rapid decrease in glutathione in both root and shoot tissue (Figs. 6.1, 6.2). A clear response was seen within two hours of treatment in root tissue, whereas shoot tissue responded less rapidly. The response to BSO was concentration dependent, and increased with the length of exposure time. At 10^{-4} M BSO, root and shoot glutathione fell to 33% of untreated tissue by 72 hours.

BSO treatments did not have any visible effects upon plant growth, or result in any damage of plant tissue over 72 hours treatment. However, longer periods of exposure to 10^{-4} BSO, resulted in severe growth deformities (Plates 6.1, 6.2). Symptoms included a general stunting and thickening of the plant stem and failure of fourth or

Fig. 6.1
BSO modulation of glutathione in maize shoot

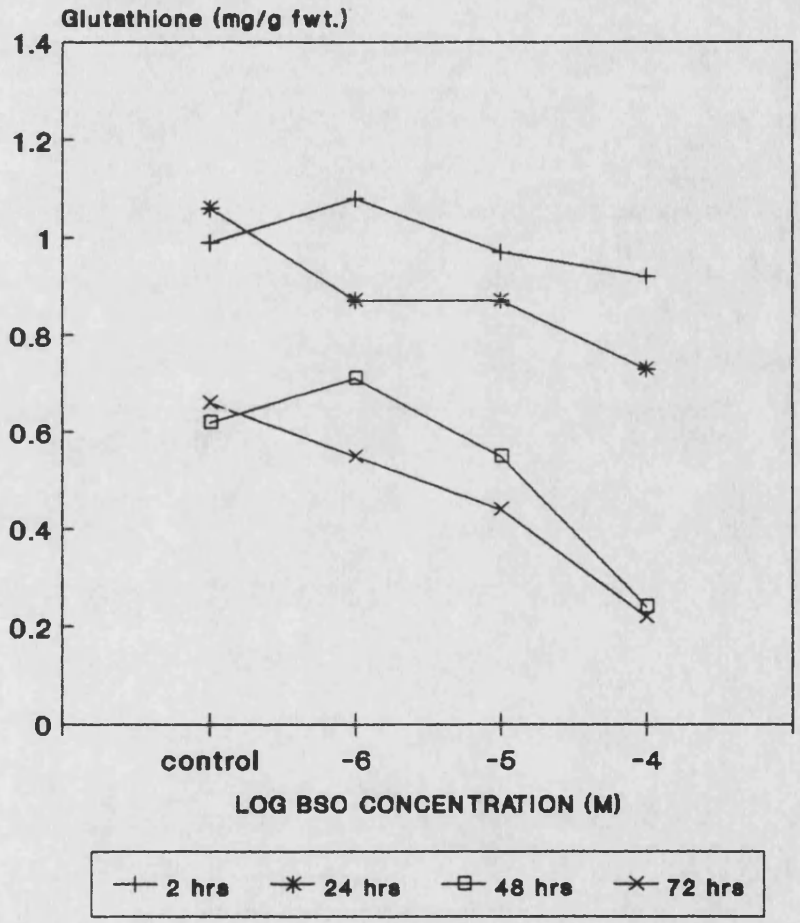


Fig. 6.2
BSO modulation of glutathione in maize root

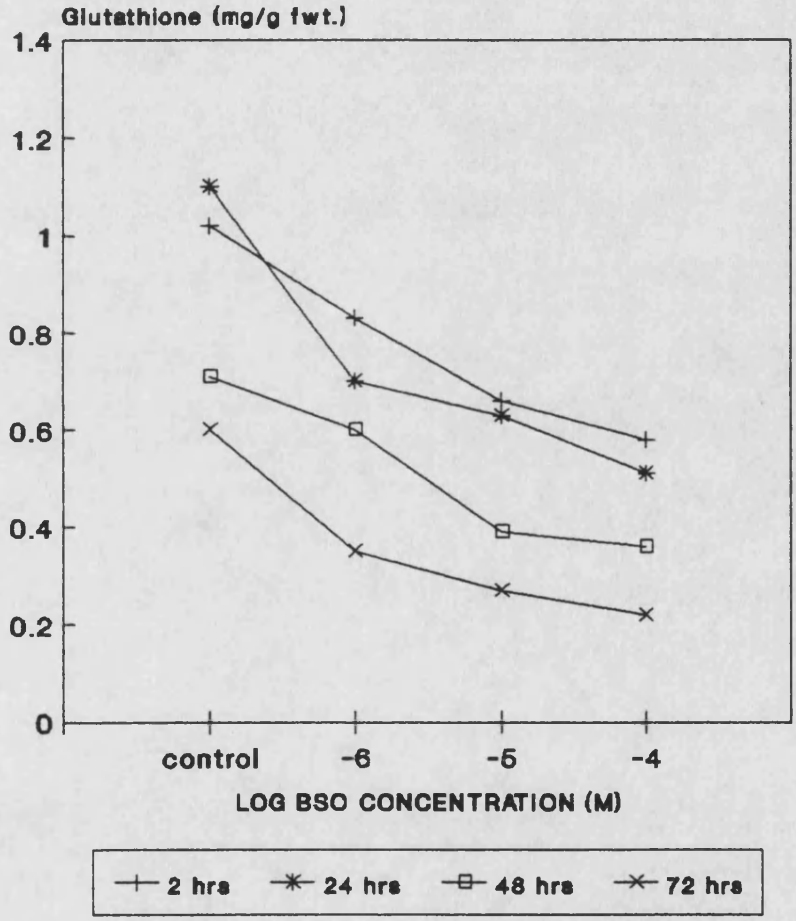


Plate 6.1 Hydroponically grown maize + 21 days with or without 10^{-4} M BSO.

Plate 6.2 Hydroponically grown maize + 21 days. Close up of 10^{-4} M BSO treatment to show leaf trapping.

CONTROL BSO

Plate 6.3 Necrosis of maize third leaf associated with 10^{-4} M BSO treatment.

Plate 6.4 Shedding of cuticle from maize leaf lamina associated with 10^{-4} M BSO treatment.

fifth leaves to emerge properly. The stunting and leaf trapping symptoms were similar to those caused by EPTC. However, plants also exhibited necrosis of immature leaves (Plate 6.3) and shedding of cuticle from the surface of leaf lamina (Plate 6.4), which do not occur with EPTC damage.

Simultaneous application of 10^{-4} or 10^{-5} M dichlormid partially counteracted the effect of 10^{-4} BSO on glutathione in shoot and root tissue (Figs. 6.3, 6.4). However, counteraction was only partial, and was less obvious in root tissue than the shoot. 10^{-5} M dichormid gave better counteraction of BSO than 10^{-4} M dichlormid, although with dichlormid treatments alone, the higher concentration gave a greater enhancement of glutathione in root tissue. It was interesting to note that dichlormid had an effect on glutathione concentrations in maize shoot tissue in this experiment - as compared to the result in section 4.3.1 where dichlormid had no effect when used as a seed dressing.

BSO proved to be ineffective in synergising the toxic effects of EPTC on maize as measured by plant growth, despite the previously observed effects upon glutathione (Tables 6.1, 6.2, 6.3). 10^{-4} M EPTC caused severe stunting of plants within three days of treatment, and resulted in a 23% reduction in plant fresh and dry weights after seven days treatment, and a 75% reduction in second leaf length. 10^{-5} M EPTC had no toxic effects on the maize plants, and caused a slight (10%) increase in all growth parameters at harvest. Treatment with 10^{-4} M BSO resulted in a 15% reduction in

Fig. 6.3 Effect of BSO and dichlormid treatments on the glutathione content of maize shoot (+ 72 hrs)

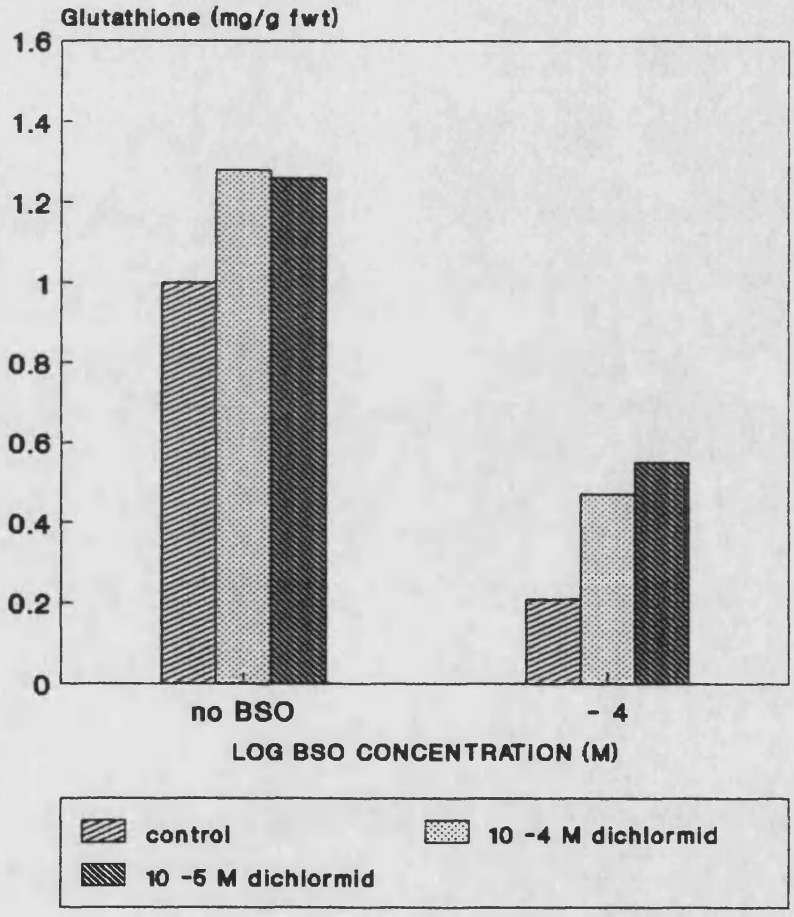


Fig. 6.4 Effect of BSO and dichlormid treatments on the glutathione content of maize root (+ 72 hrs)

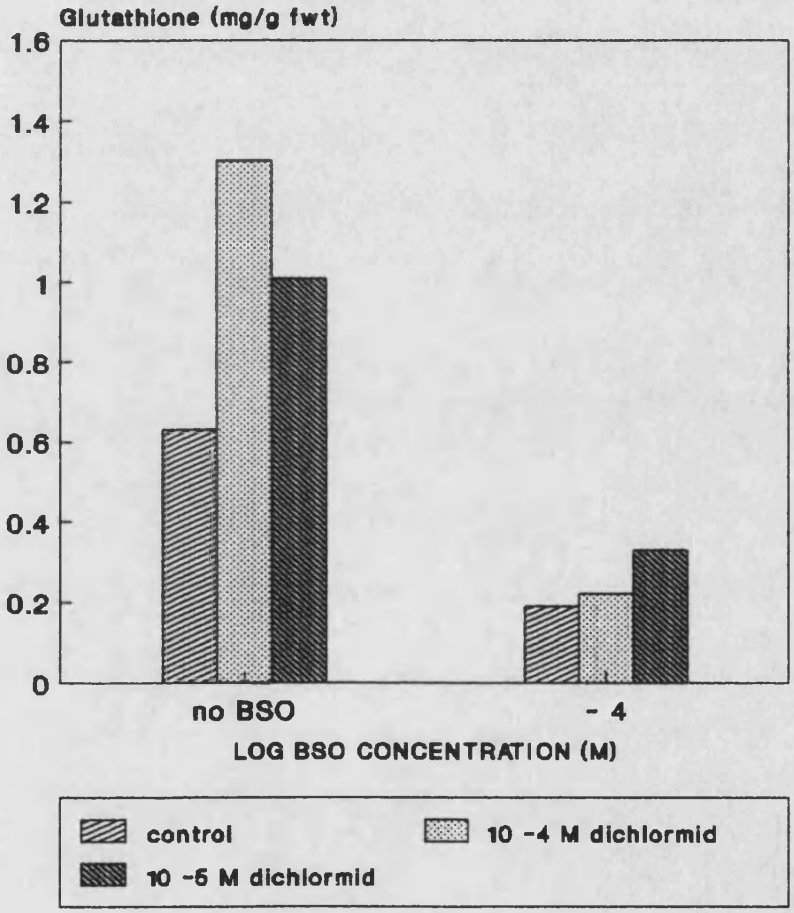


Table 6.1 Effect of BSO and EPTC treatments on maize leaf length 9 days after sowing

	Second leaf length (cm)					
BSO (M)	0		10^{-4} (at sowing)		10^{-4} (+ 48 hrs)	
	(1) $\bar{x}$	(S.E.)	$\bar{x}$	(S.E.)	$\bar{x}$	(S.E.)
EPTC (M)						
0	15.5	(0.8)	-	-	13.8	(0.9)
10^{-5}	17.2	(0.6)	13.4	(0.6)	14.7	(0.9)
10^{-4}	4.0	(0.6)	4.0	(0.4)	4.0	(0.5)

(1) n = 6

$\bar{x}$ = mean

S.E. = Standard error

Table 6.2 Effect of BSO and EPTC treatments on maize shoot fresh weight, 9 days after sowing

	Fresh weight/pot (g) (1)					
BSO (M)	0		10^{-4} (at sowing)		10^{-4} (+ 48 hrs)	
	(2) $\bar{x}$	(S.E.)	$\bar{x}$	(S.E.)	$\bar{x}$	(S.E.)
EPTC (M)						
0	2.21	(0.19)	-	-	1.86	(0.37)
10^{-5}	2.43	(0.06)	1.76	(0.20)	1.95	(0.01)
10^{-4}	1.48	(0)	1.51	(0.02)	1.49	(0.27)

Table 6.3 Effect of BSO and EPTC treatments on maize shoot dry weight, 9 days after sowing

	Dry weight/pot (g) (1)					
BSO (M)	0		10^{-4} (at sowing)		10^{-4} (+ 48 hrs)	
	(2) $\bar{x}$	(S.E.)	$\bar{x}$	(S.E.)	$\bar{x}$	(S.E.)
EPTC (M)						
0	0.208	(0.020)	-	-	0.171	(0.040)
10^{-5}	0.222	(0.004)	0.156	(0.026)	0.186	(0.006)
10^{-4}	0.140	(0.008)	0.143	(0.001)	0.127	(0.041)

(1) 3 shoots/pot

(2) n = 2

$\bar{x}$ = mean

S.E. = Standard error

plant growth. However, neither pre-treatment nor simultaneous treatment with BSO enhanced the toxicity of 10^{-4} M EPTC, or resulted in growth inhibition greater than 10^{-4} M BSO alone when applied with 10^{-5} M EPTC. Although 10^{-4} M BSO treated plants showed some stunting compared with controls, no leaf trapping or necrosis was observed over the growth period of this trial.

6.4 DISCUSSION

Although BSO was effective in reducing the glutathione content of both maize root and shoot tissue, it did not synergise the toxicity of EPTC, which is metabolised via glutathione conjugation (Hatzios and Penner, 1982). This might suggest that the glutathione content of tissue is not an important factor in determining EPTC toxicity in maize, and that glutathione-s-transferase (GST) activity is more important. If glutathione was already present in excess to requirements for EPTC metabolism, then a 60-70% reduction might not cause a critical decrease in the rate of detoxification of the herbicide. The importance of GST activity in determining the rate of EPTC detoxification has been demonstrated in some grass species by the synergistic action of tridiphane which inhibits GST activity (Dekker, 1984; Ezra, Dekker and Stephenson, 1985; Lamouruex and Rusness, 1986b). This in turn might suggest that elevation of GST activity, associated with some safener treatments, is more critical in conferring thiocarbamate tolerance on maize, than any elevation of glutathione. However, although it was demonstrated that BSO reduced glutathione concentrations when applied alone, the same

result may not have occurred in the presence of EPTC. 10^{-5} M EPTC was not toxic to maize in this trial, and in fact caused a slight increase in growth. Sub-toxic pre-treatments with herbicides have been reported to protect maize against further herbicide damage (Ezra et al., 1985; Stephenson and Ezra, 1985). Although pretreatment with 10 ppm EPTC (10^{-5} M) did not protect maize against subsequent herbicide damage, it did elevate the GSH content of roots by up to 150% (Stephenson and Ezra, 1985). Hence, some counteraction of the BSO effect on glutathione may have occurred in this trial. Further investigation in this area would help to clarify this observation.

This result might also suggest that other routes of EPTC metabolism may occur in maize which could compensate for any effects on the rate of GSH conjugation. Hatzios and Penner (1982) outlined a double N-dealkylation followed by hydrolysis as a possible alternative mechanism of thiocarbamate detoxification, but there is no further evidence to support this suggestion.

The partial counteraction of BSO induced reduction of glutathione by dichlormid suggested that BSO and dichlormid affected glutathione synthesis at separate sites. As BSO almost totally counteracted the observed enhancement of glutathione by dichlormid, it would seem probable that BSO had an effect at a later stage in the GSH biosynthetic pathway than dichlormid. This would agree with the findings of Adams, Blee and Casida (1983) that dichlormid enhanced sulphate uptake into roots and its metabolism to sulphide,

by enhancing ATP-sulphurylase activity. Rennenberg, Birk and Schaer (1982) found a general enhancement of enzymes involved in glutathione synthesis following dichlormid treatment of tobacco cells. Carringer, Rieck and Bush (1978) showed no effect of dichlormid on glutathione synthetase activity in maize roots (the enzyme following γ-glutamylcysteine synthetase in the γ-glutamyl cycle) but did find stimulation in vitro. The partial counteraction of BSO by dichlormid may have been due to a build up of metabolites preceeding γ-glutamylcysteine synthetase, because of enhanced sulphate metabolism. This could have partially overcome γ-glutamylcysteine synthetase inhibition by BSO.

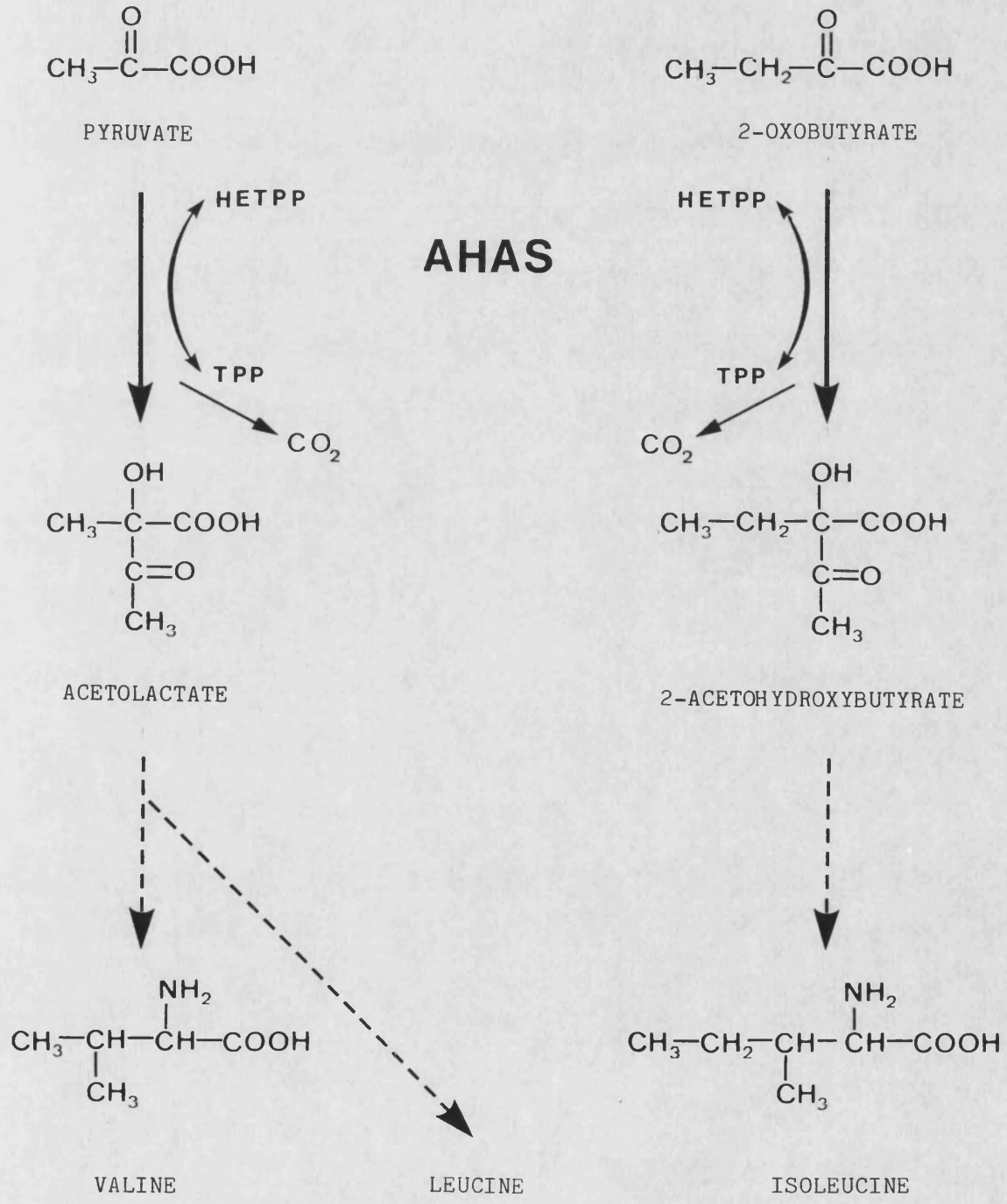
Some of the similarities between BSO and EPTC damage to maize, could indicate that glutathione depletion plays a role in EPTC toxicity. However, differences in the symptoms were also noted, which would suggest that other factors are involved. The observed effect of BSO on the plant cuticle may also indicate an important role of glutathione in cuticle biosynthesis and/or maintenance. Further investigations into this effect of BSO may prove useful to researchers in the area of plant cuticle biochemistry and physiology.

7. THE EFFECT OF NA AND DICHLORMID TREATMENT OF MAIZE ON ACETOHYDROXYACID SYNTHETASE ACTIVITY, AND INHIBITION BY CHLORSULFURON

7.1 INTRODUCTION

Acetohydroxyacid synthetase (AHAS - also referred to as acetolactate synthetase, ALS) has been identified as the site of action of the sulfonylurea (Chaleff and Mauvais, 1984; Ray, 1984) imidazolinone (Shaner, Anderson and Stidham, 1984) and sulfonanilide herbicides (Hawkes, Howard and Pontin, 1989; Schloss, Ciskanik and Van Dyk, 1988). It is involved in a combined pathway for the biosynthesis of the branched chain amino acids, valine, leucine and isoleucine and catalyses two separate reactions (Fig. 7.1). The condensation of two molecules of pyruvate to form 2-acetolactate leads ultimately to the synthesis of valine and leucine. Alternatively, 2-oxobutyrate and pyruvate are condensed to form 2-acetohydroxybutyrate, which leads to the synthesis of isoleucine. Flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP or cocarboxylase) and a divalent cation such as Mn^{2+} or Mg^{2+}) have been identified as important cofactors in these two AHAS mediated reactions (Falco et al., 1985). All three groups of herbicides have been found to be non-competitive inhibitors of AHAS with respect to pyruvate (Hawkes, Howard and Pontin, 1989). Inhibition has also been found to increase with time, as the enzyme and inhibitor form a tight complex. The herbicides cause a rapid cessation of growth in meristematic tissue, followed by chlorosis

Fig. 7.1 Role of AHAS in branched-chain amino acid biosynthesis



and necrosis of plant tissue over several weeks (Hawkes, Howard and Pontin, 1989; Ray, 1980, 1982; Shaner, Anderson and Stidham, 1984). Exogenous applications of valine and isoleucine have been found to reverse the effect of these herbicides on plant tissue (Anderson and Hibberd, 1985; Ray, 1984; Scheel and Casida, 1985; Shaner and Reider, 1986).

NA and dichlormid gave partial protection to maize against chlorsulfuron (section 3.3). These and other safeners have also been reported to provide similar protection to maize and sorghum against other sulfonylureas, and the imidazolinones, imazaquin and imazethapyr (Barrett, 1988, 1989).

In view of the reported effects of safeners on glutathione-s-transferase (GST) activity and related enzymes in treated plants (section 2.3.3(a) and results of section 4.3), the possibility that NA and dichlormid enhanced the tolerance of maize to chlorsulfuron by increasing AHAS levels or activity was considered.

Tissue culture systems have been used successfully to select for sulfonylurea resistance in tobacco (Chaleff and Mauvais, 1984) and imidazolinone resistance in maize (Shaner, Malefyt and Anderson, 1985). In both these cases, herbicide resistance was found to be associated with an altered form of AHAS which was much less sensitive to inhibition by these compounds. An altered form of AHAS has also been found to confer resistance on *E. coli* and yeast against sulfmeturon-methyl (Falco and Dumas, 1985; Yadav *et al.*,

1986). This suggested that induction of a herbicide resistant isoenzyme of AHAS might also be involved in the observed safening response in maize.

The aim of this section of work was to assess if either enhanced AHAS activity and/or induction of an isoenzyme with increased herbicide tolerance, was associated with NA and dichlormid protection of maize against chlorsulfuron. Direct competition between NA and chlorsulfuron on AHAS activity was also investigated as another possible mechanism of safener action.

7.2 MATERIALS AND METHODS

7.2.1 Plant Material

Seeds of maize (var. LG11) were washed in water for 15 minutes to remove a fungicide dressing. They were then dried, and either NA (97% w/w ai) or dichlormid (20% w/w ai) applied as a dressing at a rate of 0.5% by seed weight. Three seeds were planted per 9 cm pot in vermiculite and watered thoroughly with 50% Hoaglands solution (Hoagland and Arnon, 1950). Pots were placed in a growth cabinet (16 hours fluorescent light, 390 $\mu E\ m^{-2}s^{-1}$, 27/19°C), and watered every two days with 50 ml of nutrient solution.

7.2.2 AHAS Extraction and Assay

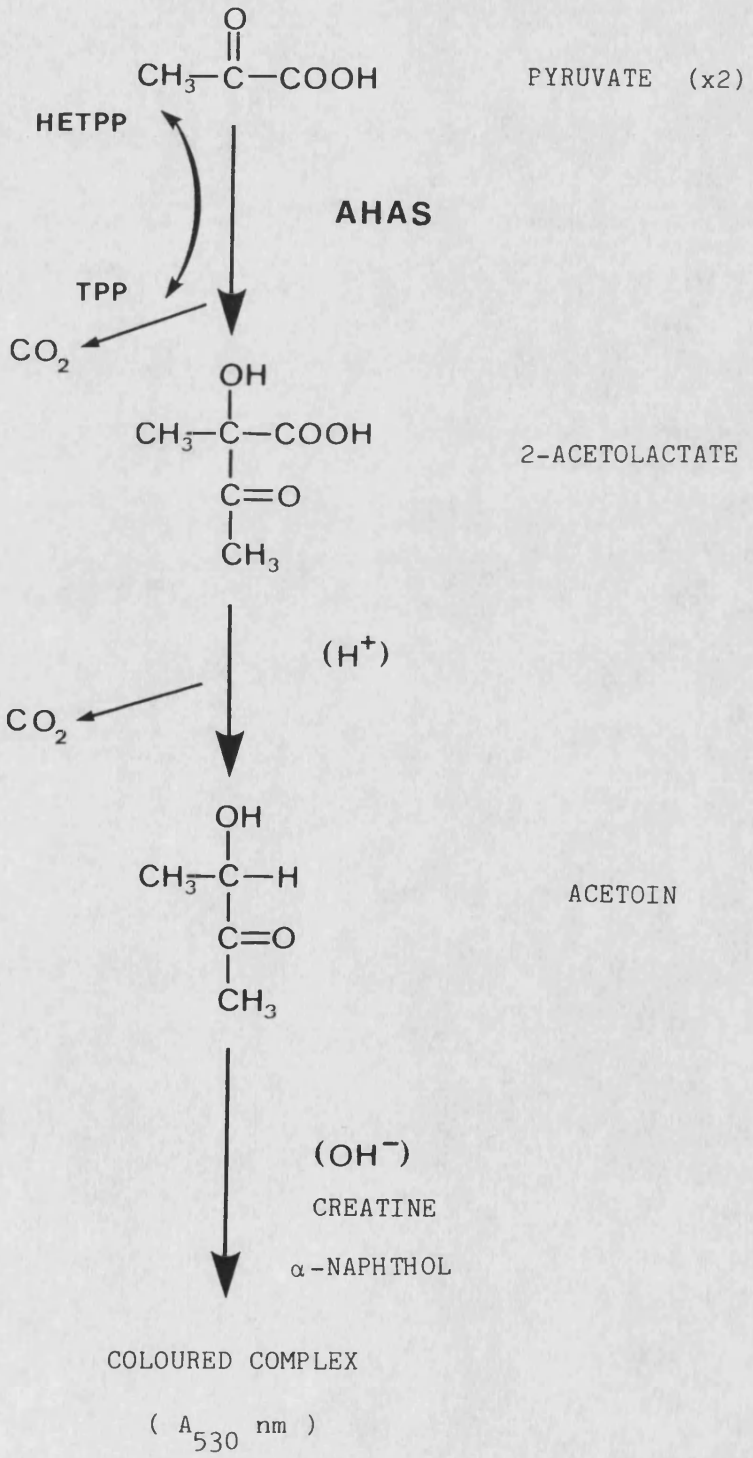
Root and third leaf tissue were used from seven day old plants

(three leaf stage). The methods used were based upon those of Chaleff and Mauvais (1984), as modified by Dr. R. Wallsgrove (Institute of Arable Crops Research, Rothamstead - personal communication). The assay depended upon the enzymatic conversion of pyruvate to 2-acetolactate followed by acid hydrolysis to form acetoin (Fig. 7.2). Acetoin concentration was then determined spectrophotometrically by the addition of α-naphthol and creatine under alkaline conditions to form a coloured complex with absorption at 530 nm (Westerfeld, 1945). The assay procedure gave a linear response to AHAS concentration for the extraction method used (Appendix 4).

(i) Leaf extraction

Approximately 0.8 g of the youngest leaves of plants was homogenised on ice in 5.5 ml of extraction buffer containing; 50 mM KH_2PO_4, 5 mM $MgSO_4$, 10 mM pyruvate, 0.5 mM cocarboxylase (TPP), 10 μM flavin adenine dinucleotide (FAD), 1 mM L-leucine, 1 mM L-valine, 10% (v/v) ethanediol and 0.05% (v/v) Triton X100. The buffer was at pH 7.5 and also contained 50% w/w polyvinyl-polypyrrolidone (PVPP) to leaf material. The crude extract was strained through four layers of muslin, and centrifuged at 11,600 g at 4°C for 8 minutes. 2 ml of the supernatant was desalted on a Sephadex G25 column equilibrated with resuspension buffer containing 50 mM KH_2PO_4 pH 7.5, 5 mM $MgSO_4$, 10 mM pyruvate, 30% (v/v) ethanediol. Desalting was carried out in a cold room at 4°C, and the resuspended enzyme extract was kept on ice until use.

Fig. 7.2 Reaction scheme for AHAS assay



(ii) Root extraction

Approximately 5 g of root tissue was ground in 10 ml of extraction buffer at 4°C, with 0.5 g PVPP and a small amount of washed sand. The resulting brei was strained through four layers of muslin, and spun at 4,000 g for 5 minutes. AHAS was precipitated from the supernatant solution with other proteins using $(NH_4)_2SO_4$ between 25% and 50% saturation. The pellet collected by centrifuging at 10,000 g (4°C) for 20 minutes, was resuspended in 2 ml of resuspension buffer and desalted as described in (i).

(iii) Assay

In a total of 650 µl, the assay solution contained; 50 mM KH_2PO_4, pH 7.5, 50 mM pyruvate, 10 mM $MgSO_4$, 250 µM cocarboxylase, 20 µM FAD, 125 µl enzyme extract, and as required 125 µl chlorsulfuron solution or H_2O. Samples were incubated at 30°C for 1 hour, after which the reaction was stopped by the addition of 125 µl of 3 M H_2SO_4. After further incubation at 60°C for 15 minutes, the acetoin content of samples was determined by the sequential addition of 125 µl 20% (w/v) NaOH, 187 µl 0.5% (w/v) creatine and 187 µl 5% (w/v) α-naphthol. After 1 hour, samples were centrifuged and absorption at 530 nm recorded.

AHAS activity was expressed as the increase in A_{530} nm compared with denatured enzyme extract controls, or as a percentage of the A_{530} nm of herbicide free controls, for chlorsulfuron treatments.

Where NA was used *in vitro*, it was first dissolved in acetone, before diluting in phosphate assay buffer. Final acetone concentration in the assay mixture was 0.25% (v/v).

7.2.3 Protein Assay

The protein content of enzyme extracts was determined using the method of Bradford (1976), with BSA as a standard.

7.2.4 Chemicals

All chemical reagents used in assays were obtained from Sigma.

7.2.5 Replication of Experiments

All results represent the average of two assay replicates carried out on at least two separate occasions.

7.3 RESULTS

7.3.1 Effect of NA and Dichlormid on Extractable AHAS Activity in Maize

At an application rate of 0.5% by seed weight, NA increased extractable AHAS activity by 1.7 to 2.0 fold in both root and leaf tissue (Table 7.1). Results were similar for AHAS activity if measured per g fresh weight of plant material, or per mg protein in

Table 7.1 AHAS levels in maize (var. LG11) root and leaf tissue following 7 days treatment with 0.5% w/w NA or dichlormid as a seed dressing

Tissue	Leaf		Root	
Treatment	A_{530} /mg P ($x10^{-3}$)	A_{530} /g fwt ($x10^{-1}$)	A_{530} /mg P ($x10^{-3}$)	A_{530} /g fwt ($x10^{-2}$)
Control	3.75	3.53	4.87	5.48
(SE)	(0.32)	(0.32)	(0.55)	(0.73)
% Control	100	100	100	100
(SE)	(8)	(9)	(11)	(13)
Dichlormid	4.09	3.74	6.07	8.43
(SE)	(0.30)	(0.36)	(1.34)	(2.25)
% Control	109	106	125	152
(SE)	(8)	(10)	(27)	(41)
NA	6.33	6.07	9.14	11.20
(SE)	(0.87)	(0.86)	(1.86)	(2.50)
% Control	169	172	188	204
(SE)	(23)	(24)	(38)	(46)

SE = standard error

the enzyme extract.

The response to dichlormid was less than that with NA. Values of AHAS activity of 1.25 to 1.5 fold were found in the root, however, error values suggested that these were not significantly different from untreated tissue. No AHAS enhancement was seen in leaf tissue following dichlormid treatment.

7.3.2 Effect of NA and Dichlormid Pretreatments on Chlorsulfuron Inhibition of AHAS extracted from Plants

To investigate the possible induction of a chlorsulfuron resistant isoenzyme of AHAS following safener treatment, inhibition curves were plotted for AHAS extracted from safened and untreated tissue (Figs. 7.3-7.6).

Neither NA nor dichlormid treatments increased the tolerance of extracted AHAS to chlorsulfuron, as measured against herbicide free AHAS activity. Hence, there was no indication of the induction of a chlorsulfuron resistant isoenzyme of AHAS being associated with safener treatment. In fact, contrary to this hypothesis, there was some indication of enhanced chlorsulfuron inhbition of AHAS from NA and dichlormid treated plants, when compared to safener free plants. I_{50} values calculated from linear regression of inhibition curves are given in Table 7.2. These suggested that AHAS from safened tissue was approximately twice as sensitive to chlorsulfuron as AHAS from untreated plants (i.e. I_{50} leaf and root

Fig. 7.3 Chlorsulfuron inhibition of AHAS from control and NA treated maize leaf tissue

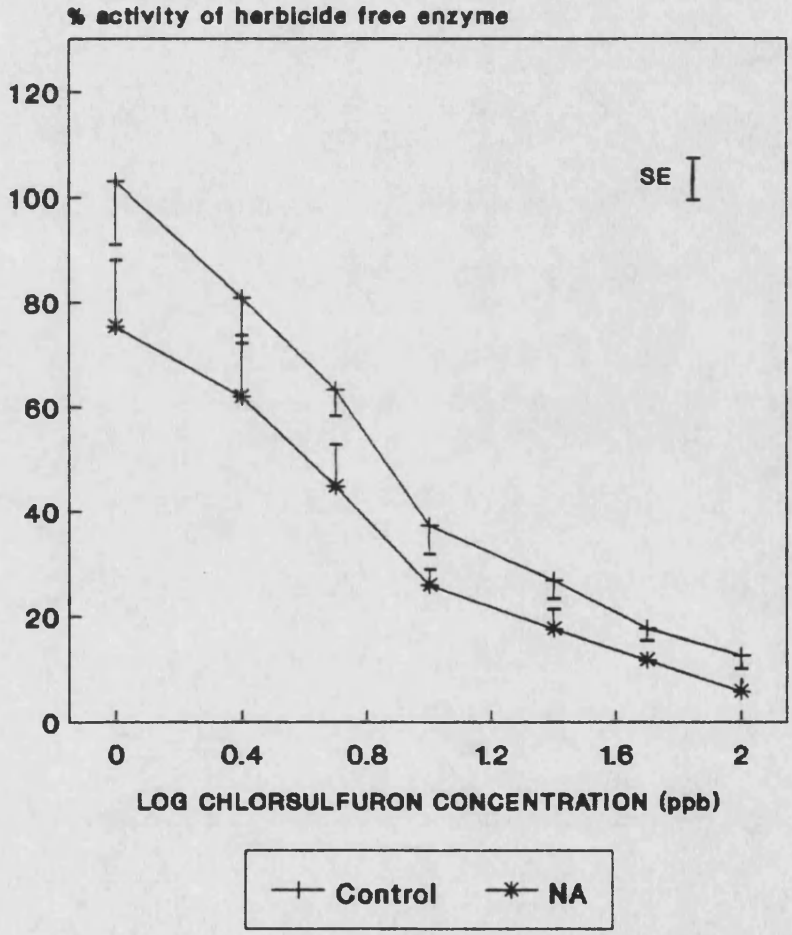


Fig. 7.4 Chlorsulfuron inhibition of AHAS from control and dichlormid treated maize leaf tissue

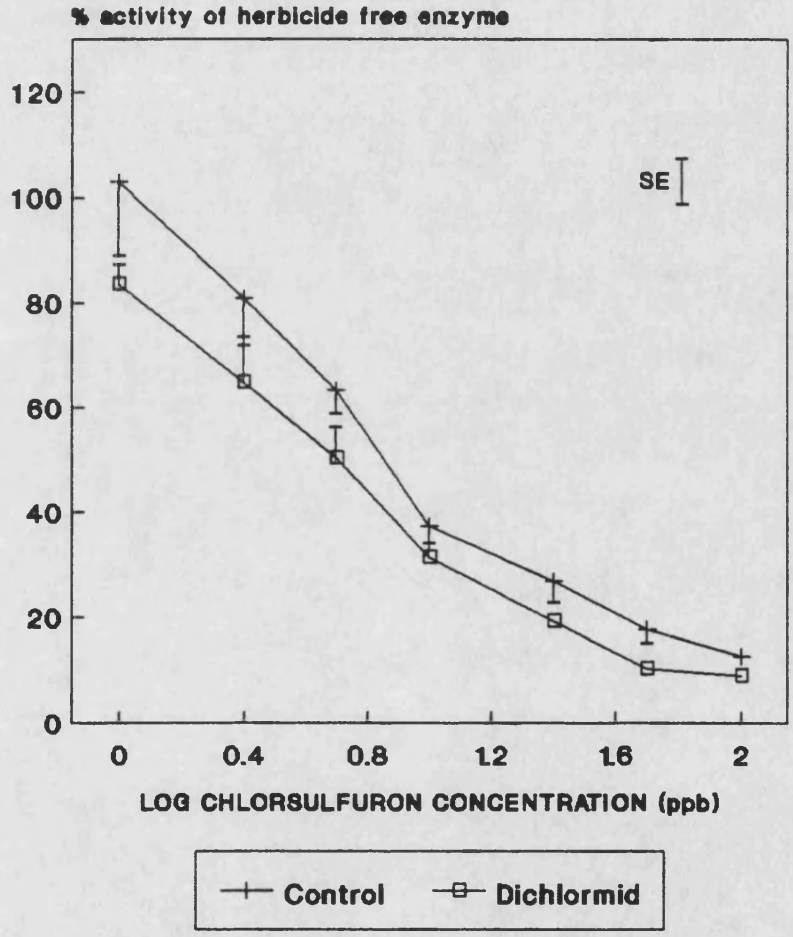


Fig. 7.5 Chlorsulfuron inhibition of AHAS from control and NA treated maize root tissue

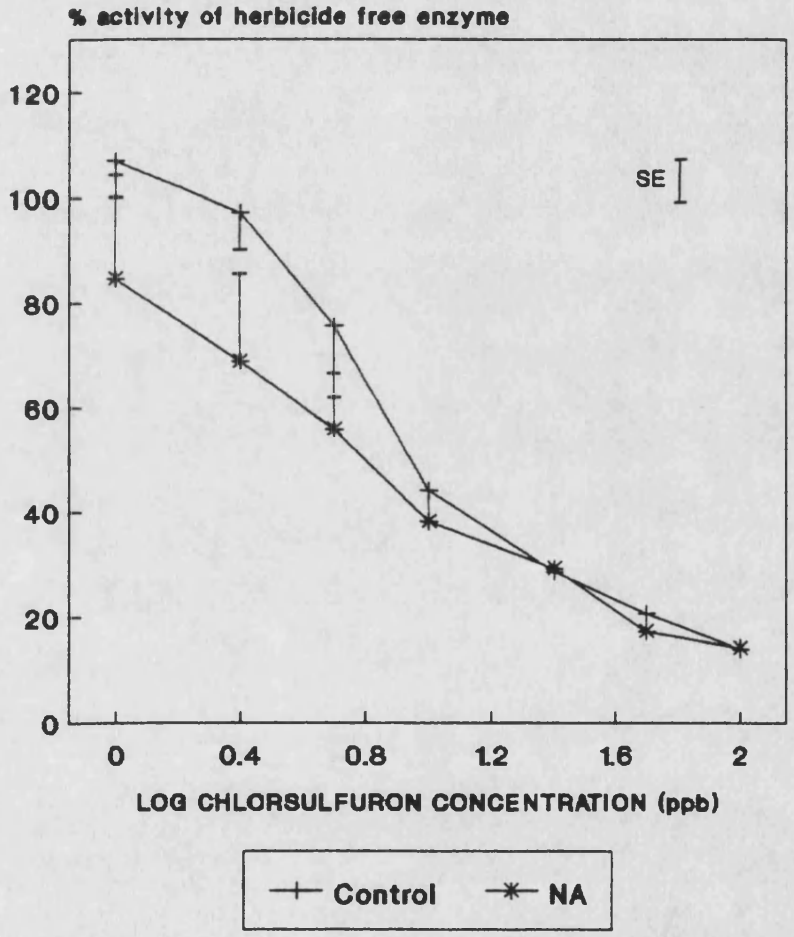


Fig. 7.6 Chlorsulfuron inhibition of AHAS from control and dichlormid treated maize root tissue

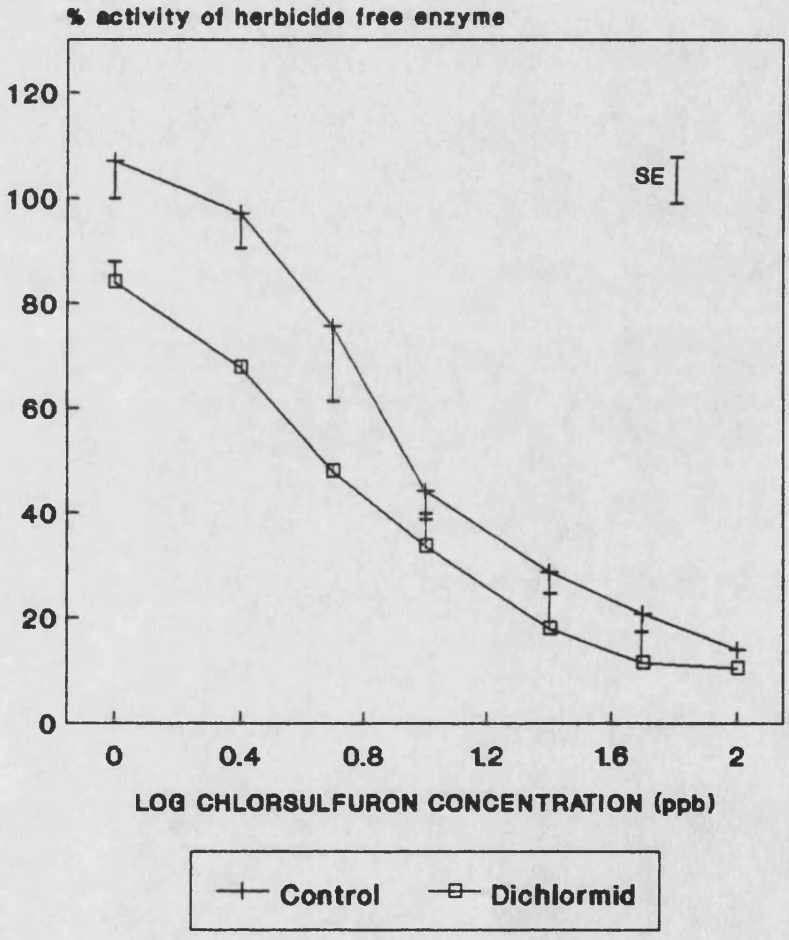


Table 7.2 Linear regression values and I_{50} for chlorsulfuron inhibition of AHAS from NA and dichlormid treated maize

Tissue	Leaf		Root	
Treatment	r	I_{50} (ppb chl.)	r	I_{50} (ppb chl.)
Control	0.955	10.00	0.954	13.40
NA	0.956	4.22	0.977	7.39
Dichlormid	0.961	5.53	0.953	5.67

Chl. = Chlorsulfuron

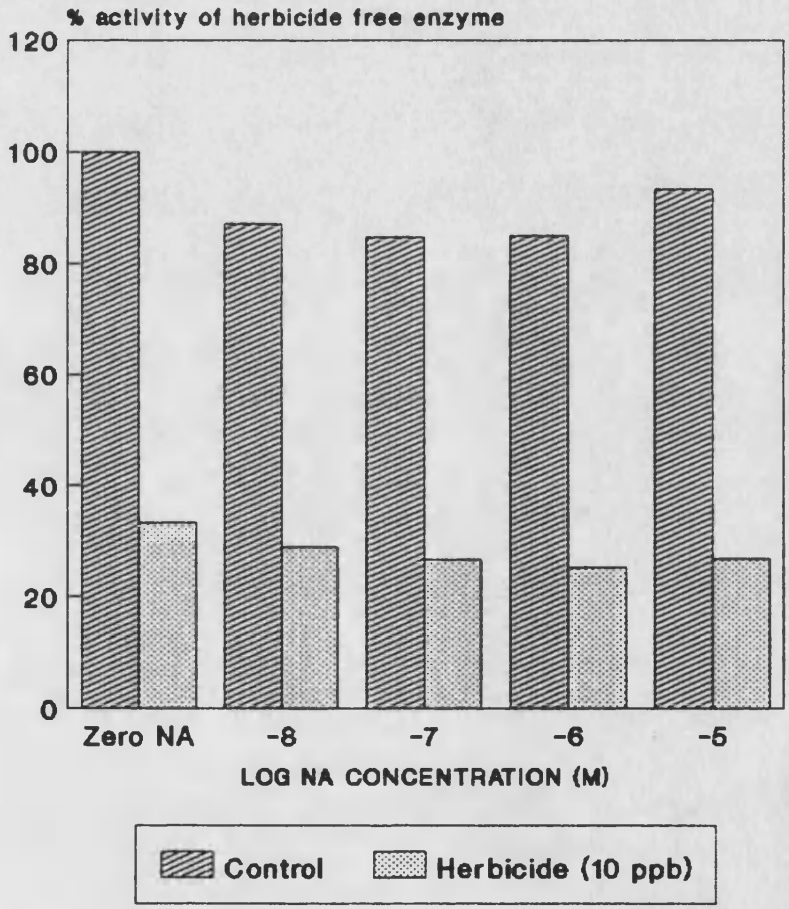
AHAS from safened plants of 5 and 6 ppb chlorsulfuron respectively, compared to 10 and 13 ppb for unsafened tissue).

7.3.3 Effect of NA *in vitro* upon chlorsulfuron inhibition of AHAS

Addition of NA *in vitro* (10^{-5} - 10^{-8} M), to AHAS extracted from unsafened leaf tissue, resulted in a 10-15% decrease in enzyme activity as compared with NA free controls (Fig. 7.7). A similar result was found with the same NA concentrations in the presence of 10 ppb chlorsulfuron.

These results suggested that NA was not having any direct competitive effect upon chlorsulfuron inhibition of AHAS in safened plants.

Fig. 7.7
Effect of NA in vitro on AHAS activity
(+/- 10 ppb chlorsulfuron)



Control value
A 630 nm = 0.341 +/- 0.055

7.4 DISCUSSION

There was some evidence to suggest that NA elevated extractable AHAS activity in maize but the response to dichlormid was less certain. For NA, AHAS activity was enhanced by a factor of X1.75 - X2.0 in both root and leaf tissue. Dichlormid had no effect upon AHAS activity in leaf tissue, but gave a slight enhancement in root tissue. However, such small changes in the level of the target site enzyme in safener treated plants would be unlikely to account for the level of protection observed, unless there was also a change in AHAS sensitivity to the herbicide.

Neither safener enhanced the tolerance of AHAS to chlorsulfuron, from treated plants. Contrary to this, there was some indication of increased AHAS sensitivity to chlorsulfuron following NA and dichlormid treatment. The I_{50} value for AHAS from safened maize tissue was approximately half that for AHAS extracted from non-safened maize. This response would tend to offset the protective effect of any increase in AHAS levels in safened plants. I_{50} values from control plants of 10-13.4 ppb chlorsulfuron were similar to 7-12.5 ppb values reported by Falco *et al.* (1985) for AHAS extracted from a range of plant species.

There was no evidence to suggest that NA altered the binding of chlorsulfuron with AHAS *in vitro*. Hence, a direct effect of NA upon AHAS binding with chlorsulfuron in safened tissue did not appear to be a likely mechanism of NA action.

Other researchers investigating the effects of safeners upon AHAS activity have tended to draw similar conclusions. Rubin and Casida (1985) reported that dichlormid enhanced the activity of AHAS in root and shoot tissue of maize var. XL25A by 25%. However, there was no change in the inhibition of the enzyme from safened tissue by chlorsulfuron. Barrett (1989) found that NA and oxabetrinil slightly enhanced the activity of extractable AHAS from etiolated sorghum shoots. However, NA, oxabetrinil and flurazole had no significant effect upon AHAS activity in root and shoot tissue of non-etiolated maize. Contrary to the results of Rubin and Casida (1985), and those reported here, dichlormid significantly reduced AHAS activity in maize tissue. Frear, Swanson and Mansager (1987) found no effect of NA on extractable AHAS from maize shoot tissue. Barrett (1989) did not investigate any changes in AHAS inhibition by herbicides following safener treatment, however, NA oxabetrinil and flurazole preserved AHAS activity in shoot tissue following imazaquin treatment, and NA had a similar effect in root tissue. NA and dichlormid had no effect upon chlorsulfuron inhibition of AHAS in vitro (Rubin and Casida, 1985; Frear, Swanson and Mansager, 1987), nor did NA, oxabetrinil, flurazole or dichlormid have any effect upon in vitro inhibition of AHAS by imazaquin (Barrett, 1989).

Hence, although safener treatment may enhance the level of AHAS activity in plants, the level of enhancement alone would appear to be insufficient to account for safening action. Direct or indirect effects of safeners on the binding of herbicides with AHAS do not

appear to be involved in safener action with the imidazolinones and sulfonylureas. Where altered forms of AHAS have been identified in plants, cross resistance between the imidazolinones and sulfonylureas has been found (Anderson and Hibberd, 1985). Thus, the lack of safener action against imazapyr (section 3.3) would also suggest that altered AHAS was not involved in the mode of safener action.

Sweetser (1985) suggested that the safening of maize against sulfonylurea herbicides was associated with enhanced metabolism via the mixed function oxidases. NA, dichlormid and cyometrinil significantly reduced the half-life of the herbicide in a range of safened plant species. Barrett (1989) found that NA, oxabetrinil and flurazole significantly increased the rate of imazaquin metabolism in maize shoots, whereas dichlormid had no effect. This correlated well with the effect of these safeners in preserving AHAS activity in imazaquin treated tissue. However, although dichlormid was the least effective of these safeners in protecting maize against imazaquin injury, some protection was observed, so other mechanisms of safener action besides enhanced metabolism are likely.

Contrary to the results of Sweetser (1985), Frear, Swanson and Mansager (1987) found no effect of NA on the rate of chlorsulfuron metabolism in maize. They stated that the same metabolic pathway for chlorsulfuron detoxification occurred in sensitive maize and tolerant wheat. This also was contrary to Sweetser (1985) who

reported a second alternative route of metabolism in maize. Some elevation of valine/leucine and isoleucine levels in meristematic tissues following NA treatment were observed, and it was suggested that this might provide transient protection against chlorsulfuron inhibition of AHAS while chlorsulfuron was detoxified.

Frear, Swanson and Mansager (1987) also found that several plant auxins: β-naphthalenacetic acid, indolebutyric acid, phenylacetic acid and indoleacetic acid, protected maize seedlings against chlorsulfuron damage. NA was reported to produce an auxin-like response in maize shoots, thus it is possible that an auxin-like response is involved in safener protection against these herbicides.

8. THE EFFECT OF NAPHTHALIC ANHYDRIDE ON THE GROWTH OF ZEA MAYS L. CELL SUSPENSION CULTURES: INTERACTION WITH THE HERBICIDES METSULFURON-METHYL AND 2,4-D

8.1 INTRODUCTION

The safening action of naphthalic anhydride is associated with a slight inhibition of early plant growth (Hickey and Krueger, 1974; Section 3.3). Investigation of the inhibitory effect of NA on actively growing cells may provide further evidence for the mode of action of this safener.

Plant cell cultures, although not necessarily meristematic in nature, provide a source of actively dividing and expanding cells on which NA action can be observed more easily than in the meristems of whole plants. Cell cultures have been used to investigate herbicide action and metabolism at a cellular level, and these responses related to effects in whole plants. Herbicide safeners have also been used in cell culture systems to investigate short-term biochemical and physiological effects, however, use of cell cultures to assess safening effects at the level of cell growth does not appear to have been reported previously.

The aim of this section of work was to assess the effect of NA on the growth of a cell suspension culture of Zea mays, and ascertain if this would provide any further evidence for its mode of action. It would also be of value to investigate if NA could provide a

safening response to a herbicide at the level of cell growth in a suspension culture. Not only would this reveal if cell culture systems could be used to screen for novel safener x herbicide combinations, but it would also indicate if the biochemical and physiological responses to safeners observed by other researchers could be related to the reversal of herbicide induced growth inhibition.

The sulfonylurea herbicide metsulfuron-methyl was chosen for investigation as its mode of action suggested that it would be effective in inhibiting cell growth (Ray, 1985). NA has also been reported to provide partial protection at whole plant level against this herbicide (Richardson, West and White, 1984).

8.1.1 Comparison of herbicide toxicity and metabolism in plants and cell cultures

Good correlations between the toxicity of herbicides in whole plants and their effects on cell cultures have been reported (Zilkah and Gressel, 1977 b; Zilkah, Bocion and Gressel, 1977). Major deviations occurred when the toxicity of photosynthetic inhibitor herbicides in plants was compared with their effects on non chlorophyllous callus tissue. Cell cultures also exhibited greater sensitivity to some compounds which was probably due to herbicide concentration being greater at the site of action in cell cultures, than when they were applied to whole plants. The growth stage of the cells at the time of treatment was found to be

important (Zilkah and Gressel, 1977a). Cells in stationary phase were more sensitive to some compounds than actively dividing cells.

Mumma and Davidonis (1983), in reviewing the use of plant cell cultures to study pesticide metabolism, concluded that metabolic pathways in cultured cells and whole plants were similar. Any differences in the metabolism of pesticides tended to be of a quantitative nature, rather than a qualitative one; e.g. Feung et al. (1978) using maize and soybean found the same hydroxylated and amino acid conjugates of 2,4-D in plants and callus tissue, but the relative amounts were altered.

However, qualitative as well as quantitative differences in metabolism between plants and cell cultures have been reported by other workers. The composition of plant cell culture media can affect herbicide metabolism. Montague et al. (1981) found that increased levels of cytokinins in media inhibited the conjugation of 2,4-D with amino acids in soybean cells. Cole and Owen (1987a) reported quantitative changes in the metabolism of chlortoluron in maize and cotton cells with changes in the organic nutrient and growth factor fraction. The level of 2,4-D was also found to affect the rate of chlortoluron metabolism in Z. mays cultures. Canivenc et al. (1988) reported both stimulatory and inhibitory effects of 2,4-D on chlortoluron metabolism in wheat cell suspension cultures, depending upon the relative time of treatment.

Biochemical differences between cell cultures and plants have been

reported to be associated with the initiation of dedifferentiated cells from differentiated plant tissue. Edwards and Owen (1986 a,b) found differences in the occurrence of isoenzymes of glutathione-s-transferase (GST) between Z. mays cell cultures and plant tissue. One isoenzyme of GST was present in maize seedlings, and this was active towards both the s-triazine herbicide atrazine and the chloroacetanilide, metolachlor. However, a cell suspension culture of the same maize variety contained two isoenzymes of GST. Neither was active towards atrazine, but both had enhanced activity towards metolachlor. The loss of GST activity towards atrazine and enhanced metolachlor activity, was associated with primary dedifferentiation of the plant tissue (Edwards and Owen, 1988).

The metabolism of herbicides by cell cultures can also alter with culture age. Cole and Owen (1988) found such a qualitative change in chlortoluron metabolism in suspension cultures of Lactuca sativa. Five year old cultures predominantly N-demethylated the 4-methyl-phenyl group of the herbicide, whereas new cultures carried out hydroxylation at an equal rate to N-demethylation.

Metabolic changes in plant cells upon the initiation of cultures and with continued culturing are not unexpected. Plants regenerated from tissue culture can exhibit inheritable phenotypic differences from the original plant. These genetic changes, which occur in mitotically dividing cells during culture, have been termed somaclonal variation (Larkin and Scowcroft, 1981), and consist of many types. These include karyotype changes (aneuploidy,

polyploidy), inter/intra chromosomal rearrangements (translocations, inversions, deletions, transpositions) and gene amplification and depletion. Somaclonal variation occurs in non-embryogenic cells (Balzan, 1978), and can increase with culture age (Lee and Phillips, 1987), though this is not true in all cultures (Edallo et al., 1981; Gresshoff and Doy, 1973).

8.1.2 Summary of Research into Herbicide Safeners Using Plant Tissue Cultures

Tissue culture systems have been used to investigate the effect of herbicide safeners at a cellular level. The research can be summarised into three categories; safener effects upon herbicide uptake, safener induced changes in metabolic pathways associated with herbicide metabolism, and safener induced antagonism of herbicide effects upon selected metabolic pathways.

Ezra, Krochmal and Gressel (1982) using maize cell suspension cultures, found that ^{14}C EPTC uptake was partially inhibited by dichlormid. The chloroacetanilide herbicide CDAA, with a similar structure to dichlormid, was less effective in reducing ^{14}C EPTC uptake, although CDAA will act as a safener against EPTC in maize. Both NA and cyometrinil enhanced ^{14}C EPTC uptake. None of the safeners had any effect on ^{14}C EPTC uptake into suspension cultured carrot cells (a species which is not safened against this herbicide). The similarity in chemical structure of dichlormid and EPTC led to the conclusion that competitive uptake of EPTC and

dichlormid into cells might partially account for the safener's action, although it was not considered a major mode of protective action. Changes in the uptake of EPTC into maize at the cellular level could not account for the protective action of NA and cyometrinil in maize.

In a different type of isolated cell system, Zama and Hatzios (1987) looked at the effect of oxabetrinil on the uptake of metolachlor into enzymatically isolated sorghum leaf protoplasts. Oxabetrinil applied simultaneously with ^{14}C metolachlor enhanced uptake of the herbicide over one hour. The safener induced enhancement of uptake was concentration dependent. It was concluded that competition for uptake at a cellular level was not a mode of safener action for oxabetrinil against metalochlor in grain sorghum.

Investigations into safener effects upon herbicide metabolism in isolated cells have centred around the glutathione and GST system. Rennenberg, Birk and Schaer (1982) found that dichlormid enhanced glutathione production in suspension cultured tobacco cells maintained under heterotrophic conditions. No such enhancement was observed with cells maintained under photoheterotrophic conditions. An increase in the level of enzymes involved with glutathione synthesis was also observed. It should be noted that tobacco is not a species which exhibits a safening response, thus the responses observed in this tissue culture system may not have reflected the mode of safener action in protecting plants from herbicide damage.

Ezra and Gressel (1982) looked at the effect of dichlormid on suspension cultured maize cells. The glutathione content of cells increased after 12 hours incubation and reached double the level in untreated cells. However, all ^{14}C EPTC was found to be metabolised by the cells within 8 hours, before any changes in glutathione content were observed. More rapid effects of herbicide and safener upon lipid biosynthesis were found, and dichlormid partially reversed the effect of EPTC upon this system. Hence, they concluded that enhancement of glutathione was not the primary mechanism of dichlormid action in maize.

Edwards and Owen (1986a) isolated two isoenzymes of GST (I and II) from maize cell suspension cultures. Both were active towards metolachlor but not atrazine. The safener fenclorim enhanced the GST II isoenzyme content of the cells, but to a lesser extent than expected from whole plants with other safeners (Lay and Casida, 1976; Mozer, Tiemeier and Jaworski, 1983). No enhancement of GST I was observed. GST II isolated from the maize cells was similar in chromatographic and kinetic behaviour to a herbicide-antidote inducible form of GST isolated from maize plants (Mozer, Tiemeier and Jaworski, 1983). Using polyclonal antibodies raised in rabbits to monitor changes in GST concentration and rates of synthesis, Edwards and Owen (1988) found that the GST substrates metolachlor and cinnamic acid raised extractable GST activity towards metolachlor in Z. mays cells, as did dichlormid. This response was not associated with de novo synthesis of enzyme.

Safener induced antagonism of herbicide effects on major metabolic processes (protein, lipid, RNA and DNA synthesis, and CO_2 fixation) has also been looked for in cell cultures.

EPTC reduced incorporation of ^3H labelled acetate into lipids of cultured maize cells (Ezra and Gressel, 1982). Dichlormid treatment enhanced ^3H acetate incorporation into lipids, and partially reversed the effect of EPTC when applied in combination. Ezra, Gressel and Flowers (1983) found that whereas EPTC partially inhibited ^{14}C acetate uptake into both polar and neutral lipids, dichlormid only enhanced uptake into neutral lipids. Hence, dichlormid did not counteract fully all the effects of EPTC on lipid synthesis.

Zama and Hatzios (1986b) used enzymatically isolated soybean leaf cells to look at the effects of oxabetrinil, cyometrinil and flurazole on CO_2 fixation, protein, lipid, DNA and RNA synthesis. Oxabetrinil and cyometrinil stimulated all processes at all but the highest concentration where their effect became inhibitory. The inhibition was not distinct and so these were not believed to be the primary sites of action. Flurazole was also stimulatory to all processes at low concentrations, but caused inhibition at a lower concentration than the other two safeners. Again it should be noted that the species used in the investigation was not one protected by these safeners at the whole plant level.

Similar work was reported by Zama and Hatzios (1987) using leaf mesophyll protoplasts isolated from grain sorghum. Oxabetrinil reduced inhibition of RNA, DNA and lipid synthesis caused by metolachlor treatment. Neither herbicide nor safener had any effect upon protein synthesis.

8.1.3 Origin of Zea mays L. var. Black Mexican Sweet Cell Cultures, and Use in Herbicide Safener Research

Black Mexican Sweetcorn (BMS) is an old standard variety of maize which has been available in the USA for over 100 years (Sheridan, 1982).

King and Shimamoto (1984) reviewing the use of maize in tissue culture found the earliest reported use of BMS as an endosperm culture established by Straus and La Rue (1954). It has proved difficult to initiate cell cultures from differentiated maize tissue. Sheridan first succeeded with BMS in 1975, establishing finely dispersed suspension cultures from an explant taken from stem sections of germinating seedlings at the first node above the hypocotyl (Sheridan, 1982). The cells have proved to be non-morphogenic, so regeneration of plants has not been possible.

This type of BMS cell culture has been frequently used by researchers. It is easier to initiate than many other varieties, and is particularly good for protoplast preparation (Sheridan, 1982).

BMS cultures have been used in a number of investigations of safener and herbicide action (Ezra and Gressel, 1982; Edwards and Owen, 1986a, 1988; Cole and Owen, 1987a).

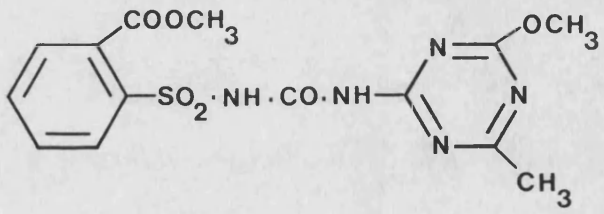
8.1.4 Metsulfuron-methyl

Metsulfuron-methyl (DPX T6376) is one of the sulfonylurea herbicides (Fig. 8.1).

Introduced by Du Pont in 1983 it was first described by Doig Carraro and McKinley(1983)as a broad spectrum cereal herbicide. It is marketed by Du Pont under the trade name "Ally" or in combination with chlorsulfuron as "Finesse" for the control of annual and perennial broad-leaved weeds in wheat, barley and oats, as pre- or post-emergence application (Hartley and Kidd, 1989).

Richardson, West and White (1984) reported a good safening response in Z. mays L. var. LG11 with NA treatment against post- emergence applications of metsulfuron-methyl. Maize proved to be tolerant to pre-emergence doses used, so no safening was observed (Richardson and West, 1984). Mersie and Foy (1984) also reported a safening response in maize. Sweetser (1985) found that NA, dichlormid and cyometrinil all reduced the half-life of metsulfuron-methyl in treated isolated leaves of maize. Tolerance of crops to sulfonylureas is believed to be associated with the rate of metabolism of the herbicides by plants (Sweetser, Schow and Hutchinson, 1982; Sweetser, 1985).

Fig. 8.1 Structure of Metsulfuron-methyl



METSULFURON-METHYL

8.2 MATERIALS AND METHODS

8.2.1 Zea mays L. var. Black Mexican Sweet: Cell line

A suspension culture of Zea mays L. var. Black Mexican Sweet (BMS) was obtained from Dr. J. Thompson, ICI Seed Support Services, Jealotts Hill. The culture was initiated in the USA in 1980, as described in section 8.1.4, and is non-morphogenic.

8.2.2 Culture Media

Cultures were maintained on Murashige and Skoog medium (MS) (Murashige and Skoog, 1962), with 2.0 mg/litre 2,4-D, and 20 g/l litre sucrose. Media pH was adjusted to 5.6 before autoclaving.

8.2.3 Culturing

Cultures were maintained in exponential growth by subculturing at seven day intervals. 10 ml of suspended cells were added to 40 ml of fresh media in a 250 ml Erlenmeyer flask. Flasks were kept on an orbital shaker at 120 rpm in a controlled environment room, maintained at 25°C and a 16 hour photoperiod under low fluorescent light.

8.2.4 Chemicals

The chemicals used in the experiments were naphthalic anhydride

(NA) (Gulf Oil) 97% w/w ai. and metsulfuron-methyl (Du Pont) technical grade.

NA was dissolved in analar acetone before addition to growth media. The final acetone concentration was 0.5% v/v, unless otherwise stated. Metsulfuron-methyl was dissolved in growth media with a few drops of NaOH to improve solubility, and further diluted with media to obtain the required concentration. This solution was filter sterilized using 0.22 μM millipore filters before addition to cell cultures. Both NA and metsulfuron-methyl stock solutions were made up freshly for each trial.

8.2.5 Time of Treatments

All herbicide and NA treatments were applied to cultures at subculture unless otherwise stated.

8.2.6 Measurement of Culture Growth

Two methods for measuring the growth of cultures were applied, although not to all experiments.

A modification of a non-destructive sampling method using settled cell volumes as described by Davis, Stolzenberg and Dusky (1984) and used by Burton and Blake (1988) was applied in initial experiments. 10 ml of agitated culture was pipetted into sterile 15 ml graduated centrifuge tubes. The settled cell volume was

recorded after 40 minutes. Cells were resuspended and returned to their original flasks under sterile conditions for continued growth. Measurements were taken at two to three day intervals.

The change in dry weight of cultures over the treatment period was also used as an alternative assessment of growth (Davis, Stolzenberg and Dusky, 1984). Initial or final flask contents were vacuum filtered, placed on pre-weighed aluminium foil sheets, and air dried for 12 hours. Samples were then dried for 48 hours in an oven at 85°C before weighing.

Because of the difficulty and risk of contamination of the settled cell volume technique, dry weights were used for most experiments.

8.2.7 Assessment of Cell Viability

The effect of herbicide and safener treatments upon cell viability at five days was assessed using a combination of fluorescein-diacetate (FDA)/propidium iodide (PI) double staining (Jones and Senft, 1985) and FDA/Evans Blue double staining techniques. Cells were viewed using a Zeiss microscope with a combination of ultraviolet and visible light sources.

Fluorescein-diacetate (FDA) is lipid soluble, and diffuses rapidly across cell membranes into cells, where it is converted to fluorescein by esterases. Fluorescein is less lipid soluble and so accumulates within cells with intact membranes. These fluoresce

green under ultraviolet light (Heslop-Harrison and Heslop-Harrison, 1970). Propidium iodide is excluded from cells with intact membranes, but binds to the nucleic acids of cells with damaged membranes producing a red fluorescence under ultraviolet light (Jones and Senft, 1985). Evans Blue is excluded from viable cells (Widholm, 1972). Used in combination with FDA under a combination of UV and visible light sources, Evans Blue produced good contrast between dark stained non-viable cells and green fluorescent viable cells.

8.2.8 Calculation of the Generation Time of Cultures

Settled cell volume values were used to calculate the generation time (g) of the cells, under some of the treatment conditions, according to the equation:

$$g = \frac{0.693\ (t_2 - t_1) \times 24}{(\log_{10} N_2 - \log_{10} N_1) \times 2.3}$$

where t = time in days

N = culture volume

g = generation time (hrs)

8.2.9 Replication of Experiments

All results of growth experiments are the mean of at least three replicates, or two replicates on two separate occasions.

8.3 RESULTS AND DISCUSSION

8.3.1 Growth of Black Mexican Sweetcorn Cultures

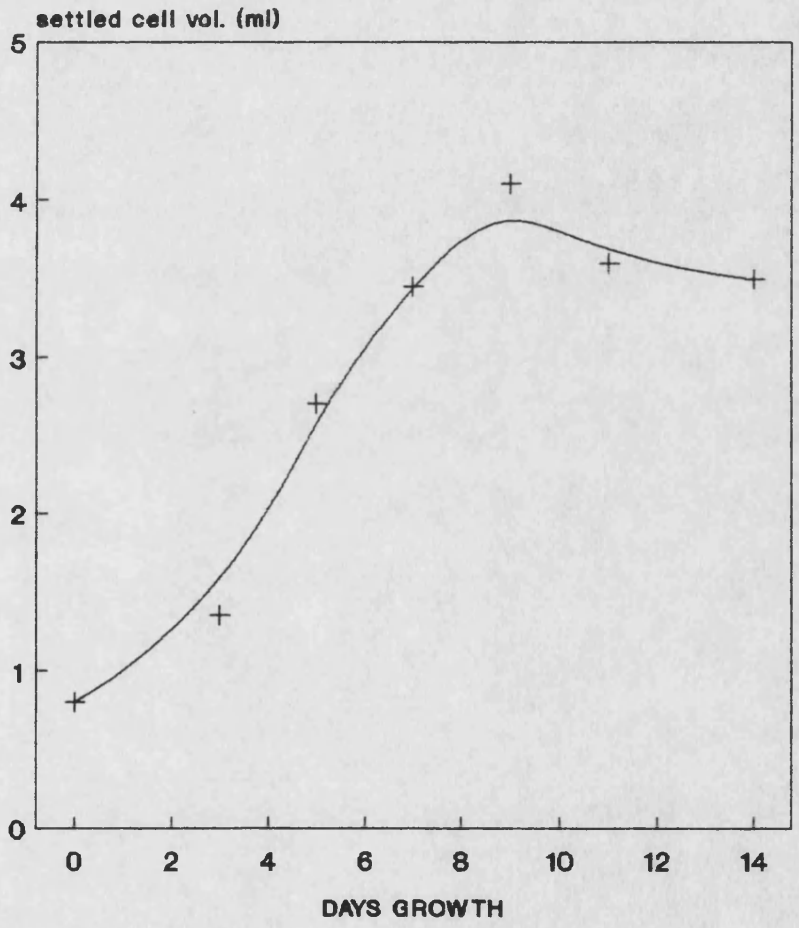
An initial experiment defined the growth of BMS cell cultures under standard conditions for a 14 day period, using settled cell volumes as a parameter of growth.

Settled cell volume increased to a maximum at day 9. Cells then entered stationary phase and the volume decreased slightly to day 14 (Fig. 8.2). The cultures developed a yellow/brown colour and a slight smell from day 11/12. Microscopic examination of the culture and subculturing into nutrient broth gave no indication of bacterial contamination associated with the colour change and smell. It was concluded that the cells had started to degenerate by day 12. Fluorescein-diacetate staining (FDA) of day 12 cultures revealed a rapid development of fluorescence in media around the cells, whereas the cells showed poor staining. This indicated a build up of esterases in the culture media and/or a loss of cell membrane integrity with age, allowing fluorescence to leak out.

Maximal growth rate was observed between days three and seven, and gave a generation time of 73 hours.

Examination of day five cultures using a combination of fluorescence and light microscopy, with FDA, PI and Evans Blue stains, revealed that the cultures consisted of clumps of two or

Fig. 8.2
Growth of BMS cell culture
(measured by settled cell volume)



three cells to groups of 30 or more. Individual cells were approximately 40 µM in size, with some elongated and enlarged cells of 80-160 µM (Plates 8.1, 8.2). Viability of day five cultures was 90-95% (Plates 8.3, 8.4).

It was concluded that the effect of NA and metsulfuron-methyl treatments on BMS cell growth should be assessed within 9 to 10 days of subculture, while cultures were still growing actively.

8.3.2 Response of BMS Cultures to NA Treatment

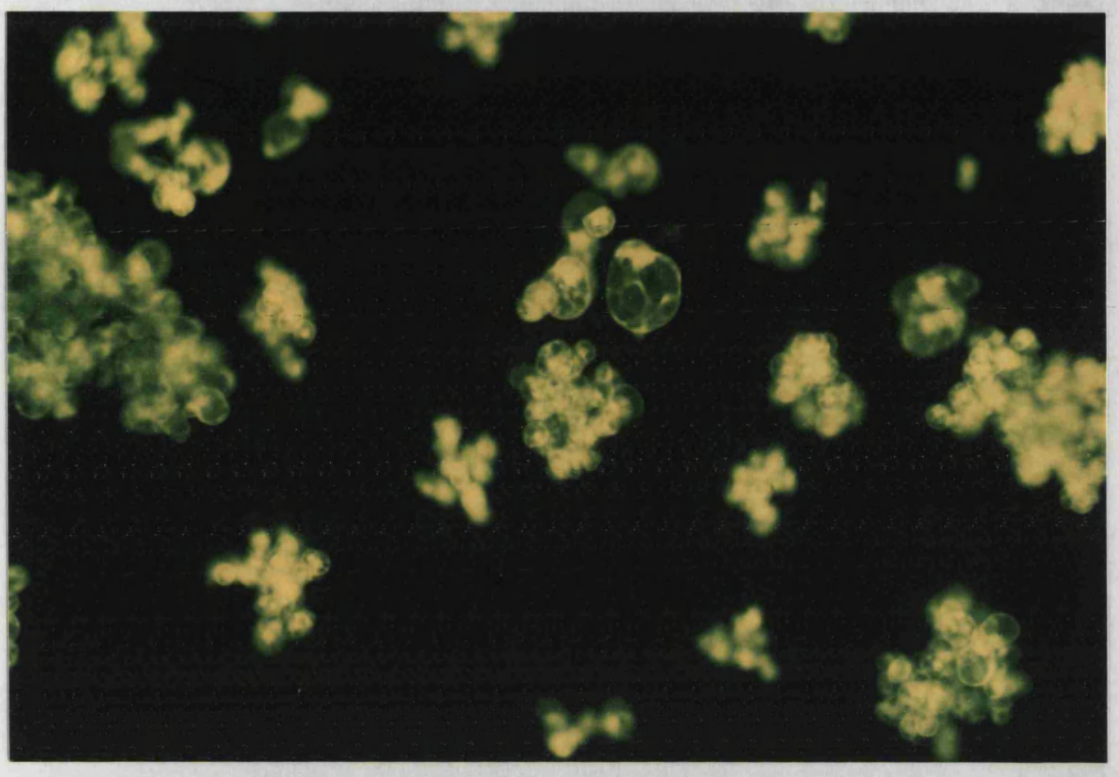
NA inhibited the growth of BMS cultures when applied at concentrations above 10^{-6} M at subculture (Figures 8.3, 8.4, 8.5).

The response to NA treatment was more pronounced with time (Fig. 8.3) and was clearly evident in the final dry weight of cultures (Fig. 8.4). At 5×10^{-6} and 10^{-5} M concentrations, NA only partially inhibited cell growth, and significantly increased cell generation time (Table 8.1). 5×10^{-5} M NA applied at the time of subculturing proved to be very phytotoxic. Cultures showed no increase in cell volume (Fig. 8.4) or dry weight (Fig. 8.3) over the treatment period.

PI/FDA/Evans Blue staining revealed a high percentage of cell death at 5×10^{-5} M by day five (Plates 8.5, 8.6). However, no significant change in cell viability occurred with NA at concentrations of 2.5×10^{-5} M and below (Plates 8.7, 8.8).

Plate 8.1 BMS suspension culture + 5 days growth: no treatment. (FDA stained, X112 magnification).

Plate 8.2 BMS suspension culture + 5 days growth: no treatment. (FDA stained, X112 magnification).



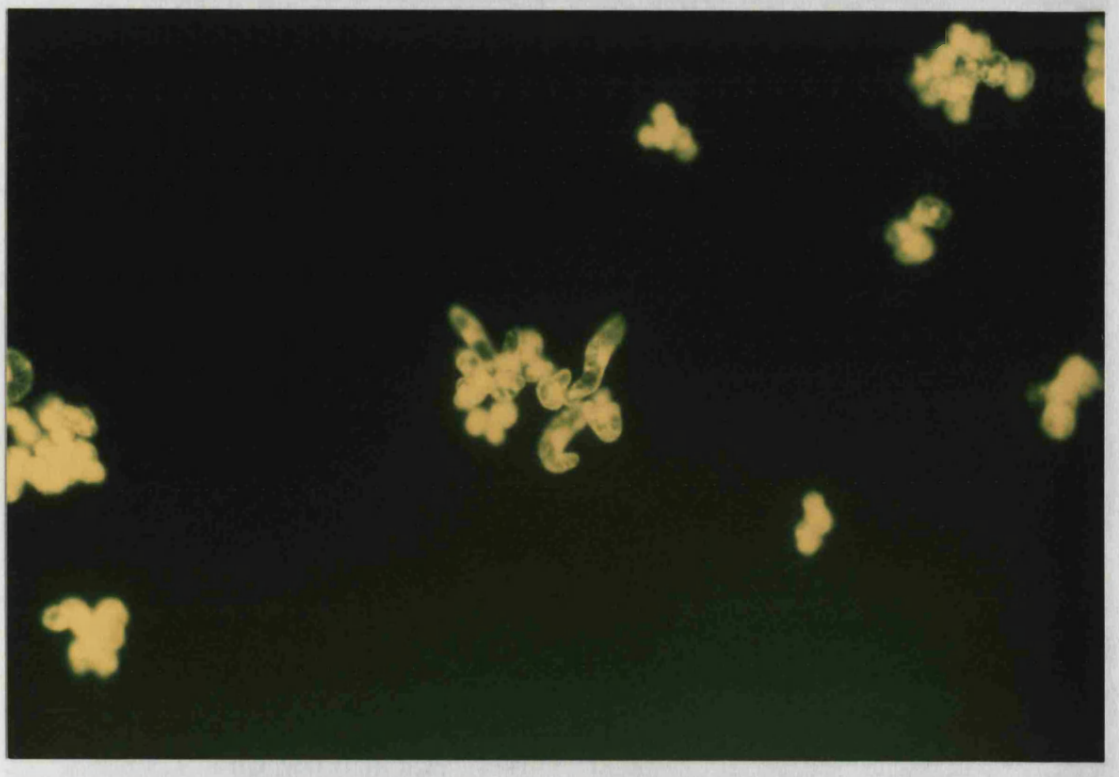
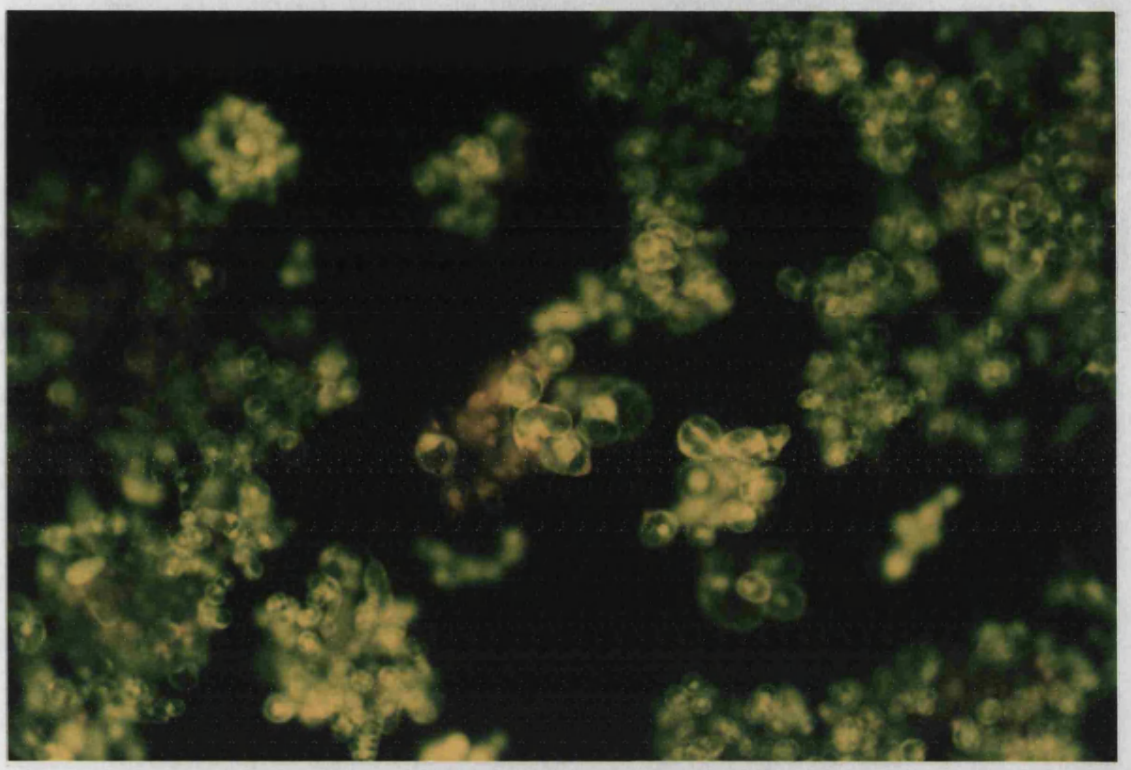


Plate 8.3 BMS suspension culture + 5 days growth: no treatment. (FDA + PI stained, X112 magnification).

Plate 8.4 BMS suspension culture + 5 days growth: no treatment. (FDA + EB stained, X112 magnification).



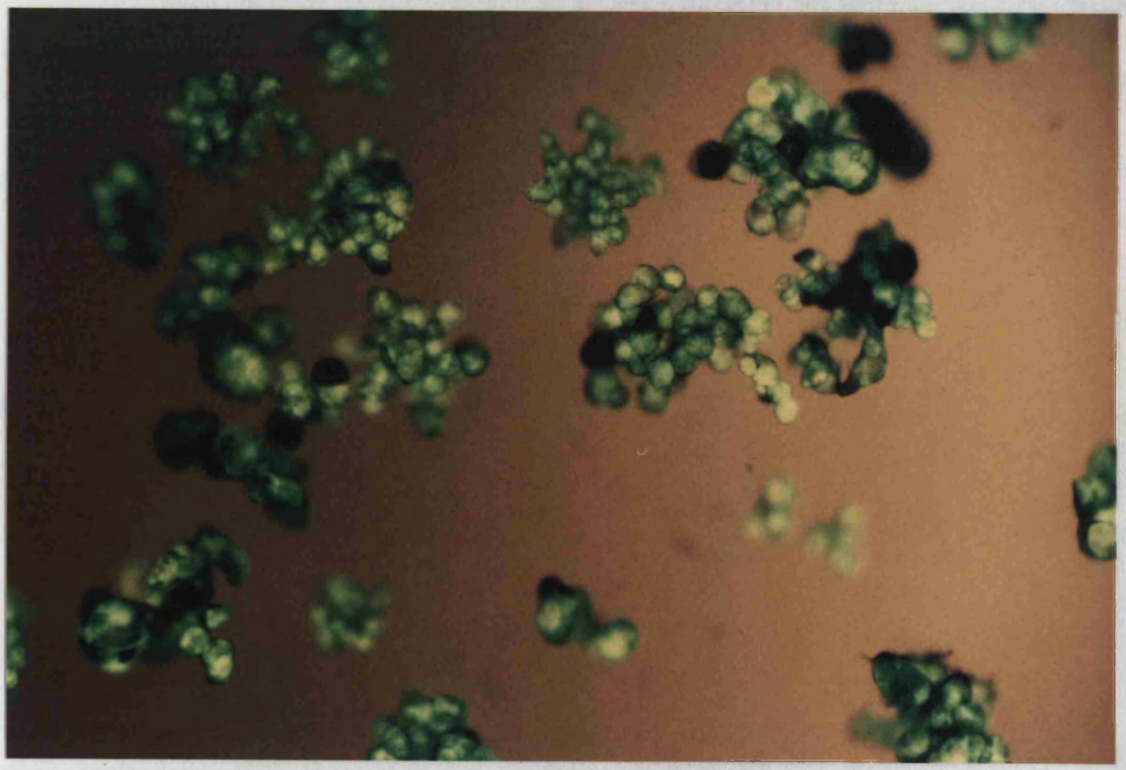


Fig. 8.3
Effect of NA concentration on BMS culture growth (settled cell volume)

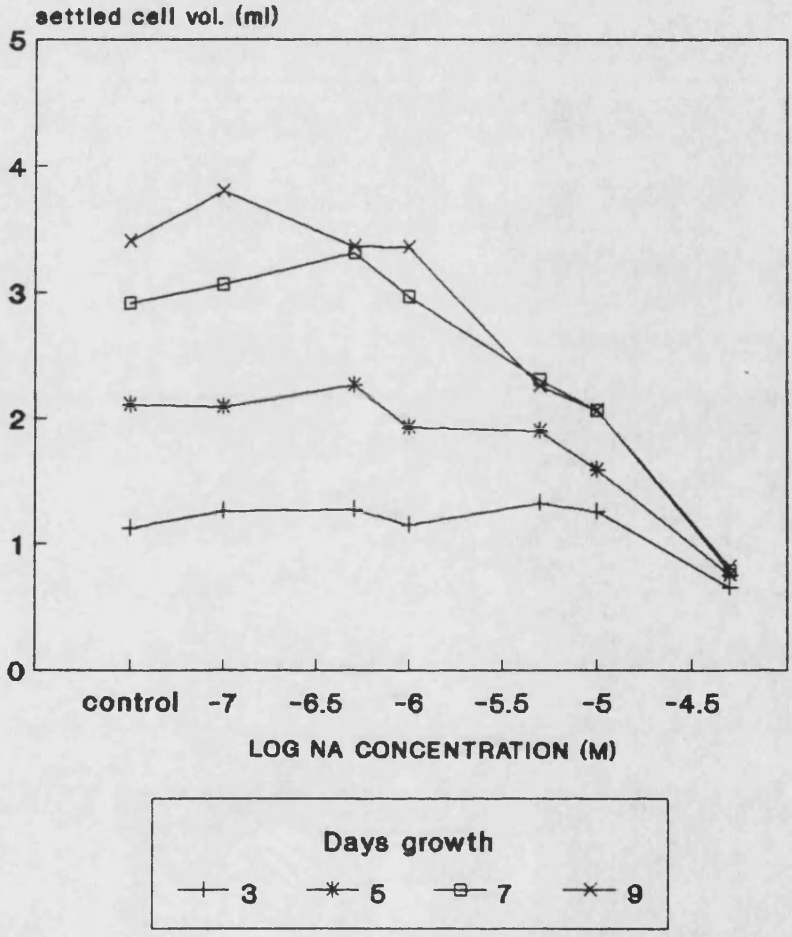


Fig. 8.4
Effect of NA on BMS culture dry weight
+ 9 days growth

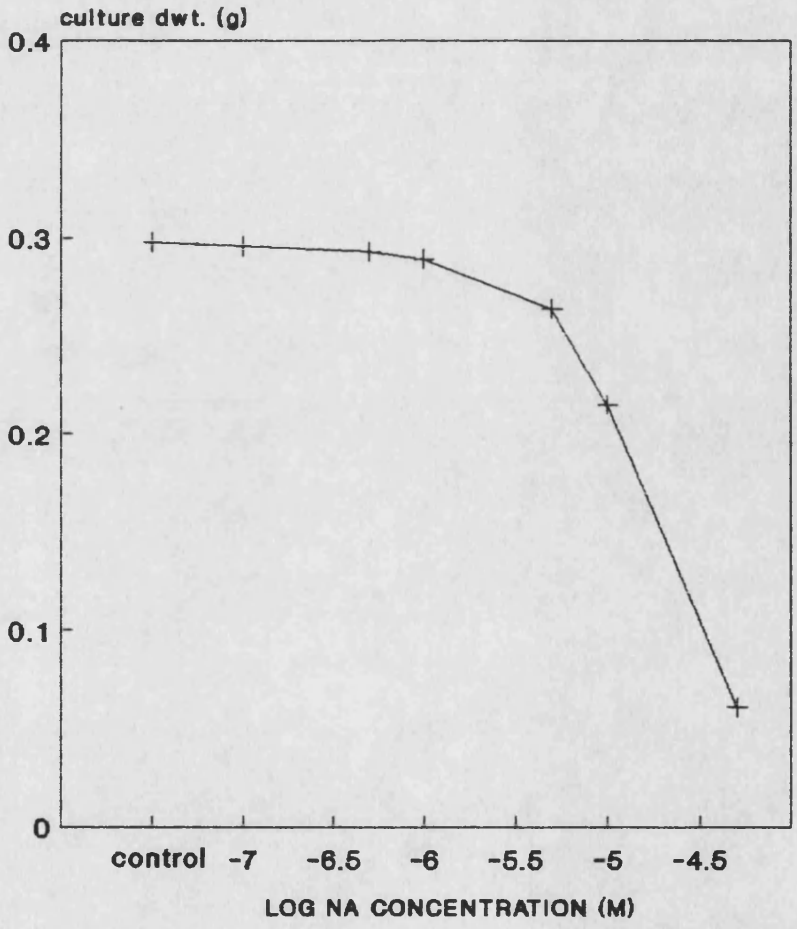


Fig. 8.5
Effect of NA on BMS culture growth with time (settled cell volume measurements)

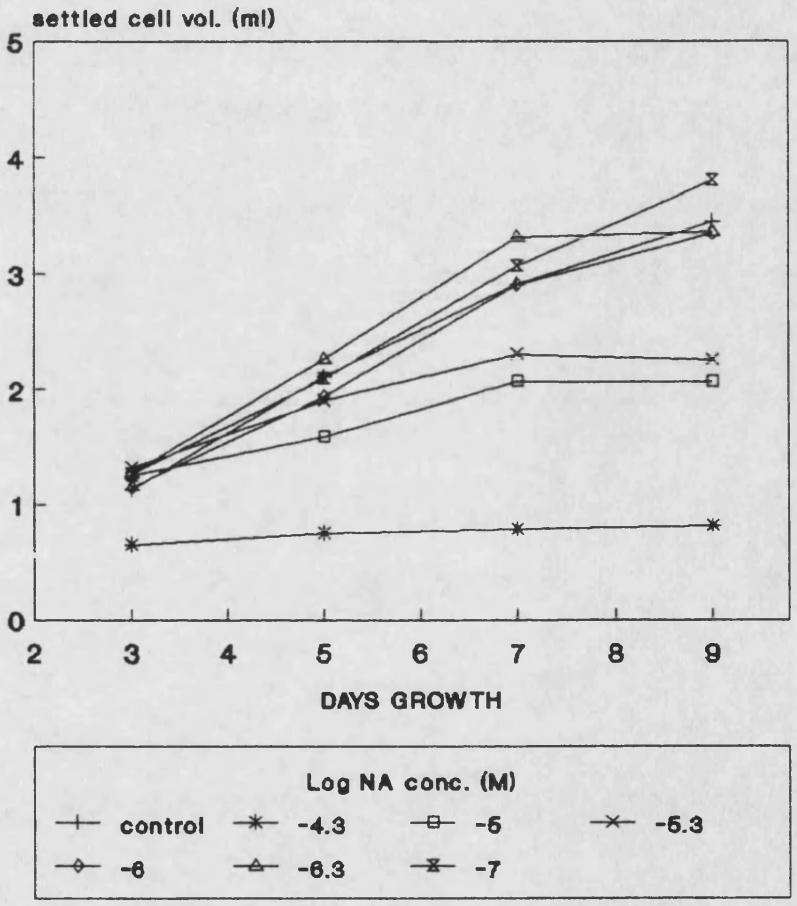


Table 8.1 Effect of NA applied at subculture on the generation time of BMS cells between days three and seven (growth measured by settled cell volume).

Concentration NA (M)	Generation time (hrs)
0	69.7
10^{-7}	74.9
5×10^{-7}	69.5
10^{-6}	72.1
5×10^{-6}	119.5
10^{-5}	133.3
5×10^{-5}	(366)(1)

(1) Only slight growth occurred.

The response of BMS cultures to NA was found to be dependent upon the time of treatment after subculturing. Severe inhibition of growth occurred with 5 x 10^{-5} M NA applied at subculture, but only partial inhibition occurred when this was applied 72 hours later (Fig. 8.6). Cell viability was also higher at five days with the later application (Plate 8.9).

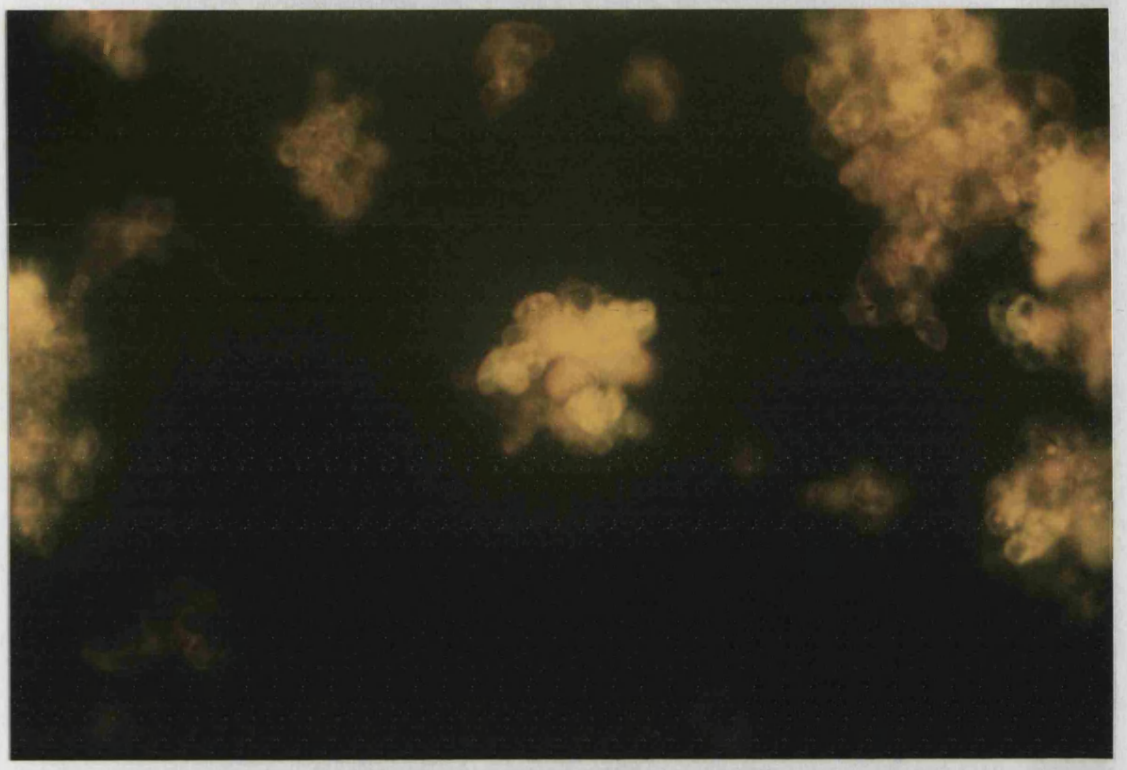
The change in sensitivity of the BMS culture to 5 x 10^{-5} M NA between days 0 and three, may in part have been due to a doubling in culture mass over this period (Fig. 8.6). As previously mentioned, FDA staining of cultures suggested that 2.5 x 10^{-5} M NA applied to cultures at day 0 did not cause noticeable damage to cells. By day three, the ratio of cell mass to NA concentration with 5 x 10^{-5} M NA would have reached the same level as 2.5 x 10^{-5} M at day 0. This may have accounted for its reduced phytotoxicity.

The growth phase of the culture may also have had an effect upon the sensitivity of the cells to NA. Zilkah and Gressel (1977a) noted that the growth regulator dikegulac had a far greater inhibitory effect upon growth of Chrysanthemum and Cirsium callus growth if applied to stationary phase cells than exponentially growing cells. NA applied at subculture may have affected cells during the lag-phase more severely than actively growing exponential cells at day three.

Early exponential growth of cultures involves a high degree of cell division compared to the later stage when cell expansion

Plate 8.5 BMS suspension culture + 5 days growth: 5 x 10^{-5} M NA applied at day 0. (FDA + PI stained, X112 magnification).

Plate 8.6 BMS suspension culture + 5 days growth: 5 x 10^{-5} NA applied at day 0. (FDA + EB stained, X112 magnification)



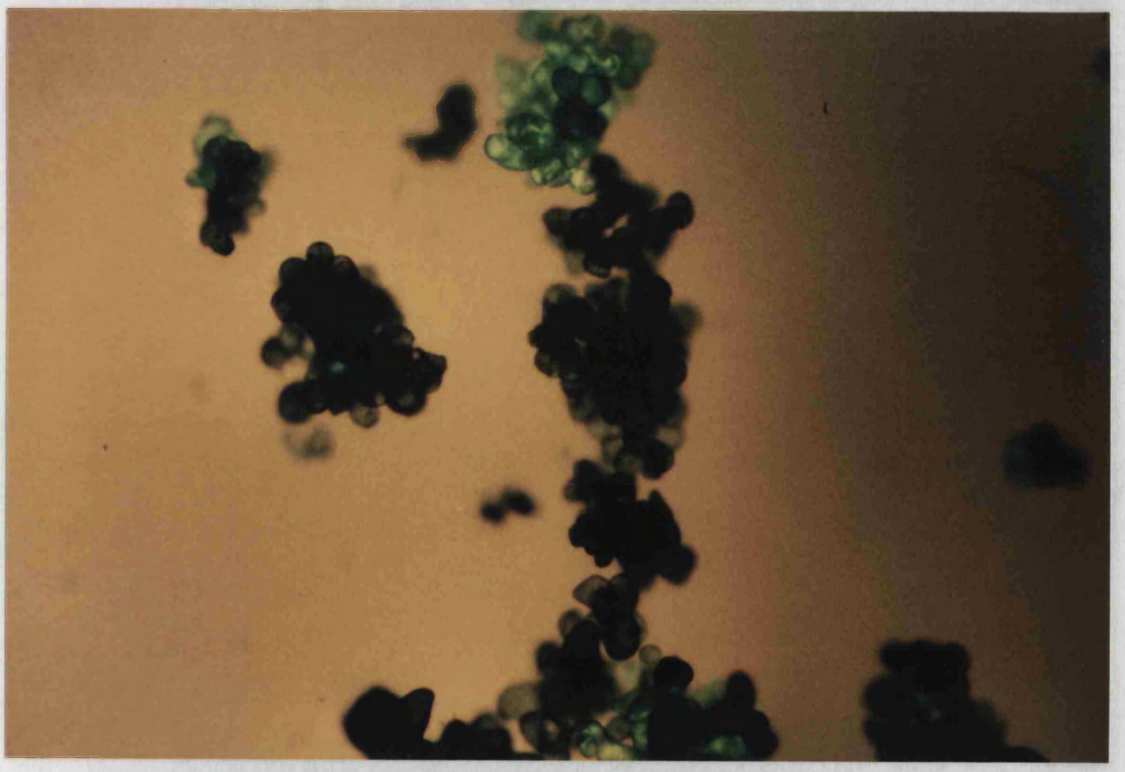


Plate 8.7 BMS suspension culture + 5 days growth: 2.5×10^{-5} M NA applied at day 0. (FDA + EB stained, X112 magnification).

Plate 8.8 BMS suspension culture + 5 days growth: 10^{-5} M NA applied at day 0. (FDA + EB stained, X112 magnification).

Plate 8.9 BMS suspension culture + 5 days growth: 5×10^{-5} M NA applied at day 3. (FDA + EB stained, X112 magnification).

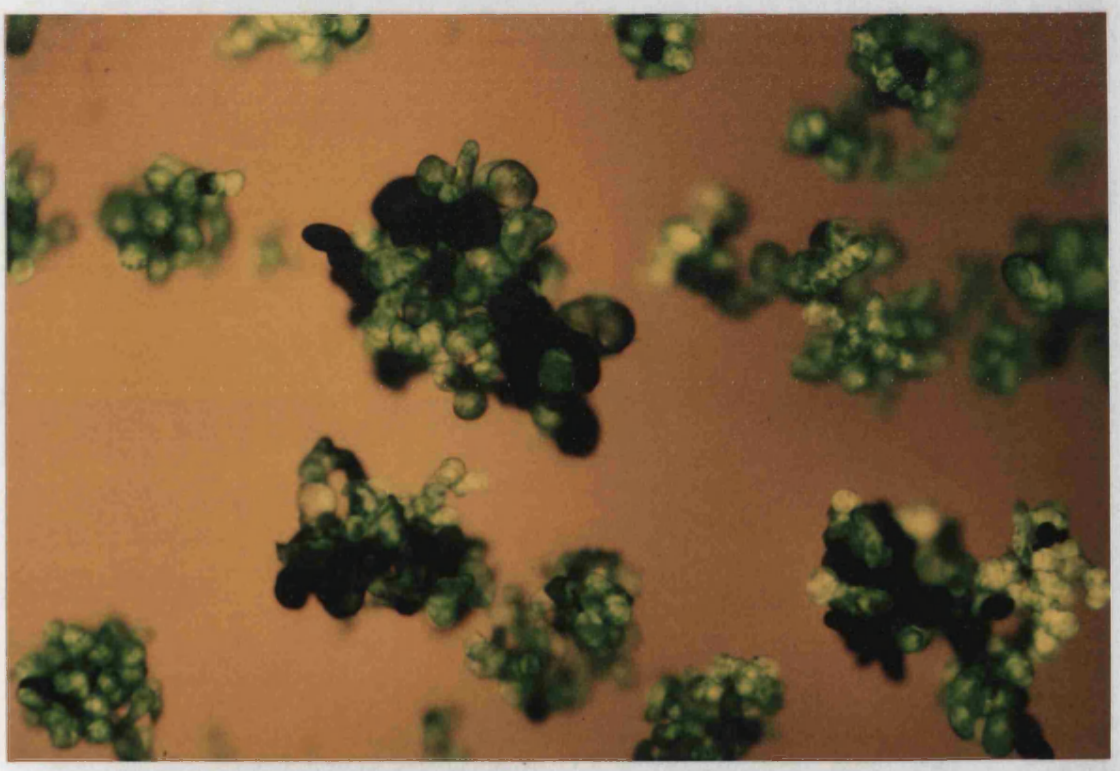
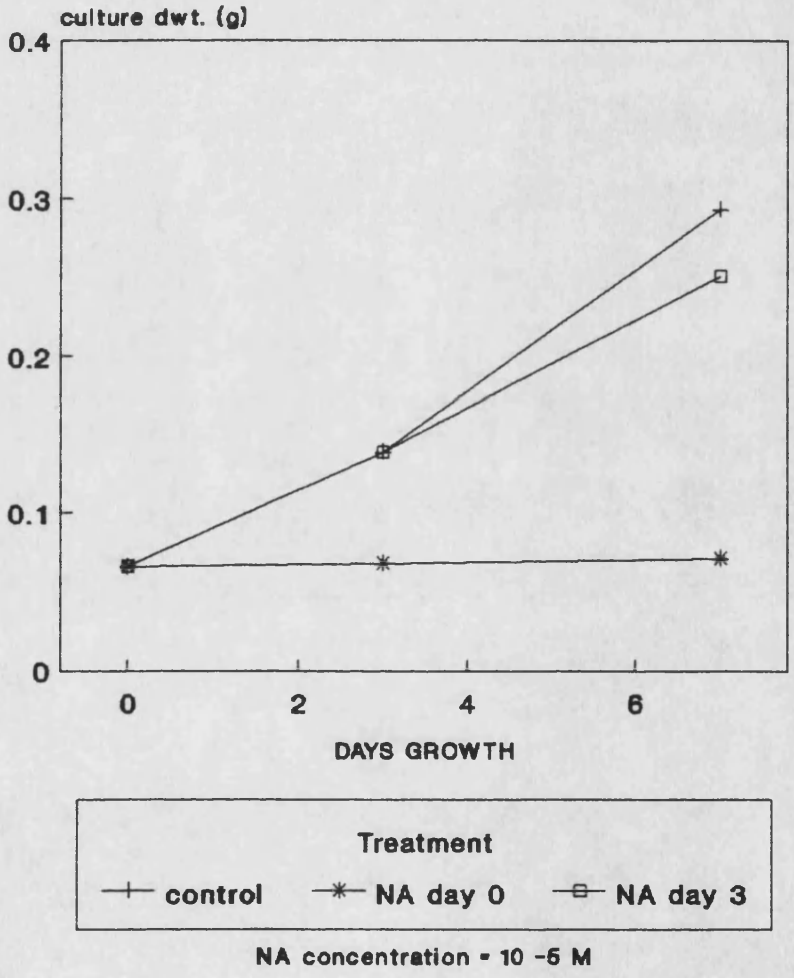


Fig. 8.6 Change in effect of NA on BMS culture growth with time of application after subculture



predominates (Oluf and Shyluk, 1981). The stunting effect of NA, or inhibition of cell growth, may have been associated with an inhibition or slowing down of cell division, rather than effects on cell elongation.

8.3.3 Response of BMS Cultures to Metsulfuron-Methyl

Reports by Richardson, West and White (1984), Mersie and Foy (1984) and Sweetser (1985) indicate that Zea mays L. is sensitive to metsulfuron-methyl. Previous results (section 7.3 in this thesis) also indicated that sulfonylureas were potent inhibitors of acetohydroxyacid synthetase (AHAS) at concentrations as low as 5 ppb. Thus, in initial investigations into the response of BMS cultures to metsulfuron-methyl, herbicide concentrations of 0.1 to 1000 ppb were used.

The response of BMS to metsulfuron-methyl was dependent upon the time of herbicide treatment (Tables 8.2, 8.3). Applied at subculture, almost total inhibition of growth occurred at concentrations as low as 0.1 ppb. However, treatment of cultures after three days gave only partial inhibition of growth at concentrations between 10 and 1000 ppb.

Inhibition of culture growth caused by 1 ppm metsulfuron-methyl decreased from 87% of control when applied at subculture to only 5% of control, when applied 72 hours later (Figure 8.7, Plates 8.10, 8.11).

Table 8.2 Effect of metsulfuron-methyl applied at subculture on BMS culture growth

Metsulfuron-methyl (ppb)	Cell volume[1] (ml)	Dry weight[1] (g)
0	3.6	0.315
0.1	*	*
1	0.85	0.076
5	0.85	0.080
10	0.95	0.081
50	00.95	0.084
100	0.80	0.077
500	1.0	0.091
1000	*	*

(1) Culture growth at day 10.

Table 8.3 Effect of metsulfuron-methyl applied at day three on BMS culture growth

Metsulfuron-methyl (ppb)	Cell volume[1] (ml)	Dry weight[1] (g)	
0	3.25	0.289	(0.310)[2]
0.1	3.3	0.281	*
1	3.2	0.274	(0.309)
5	*	*	*
10	2.1	0.233	(0.308)
50	*	*	*
100	2.7	0.260	(0.302)
500	*	*	*
1000	*	*	(0.268)

(1) Culture growth at day 10.

(2) ( ) dry weights taken only

The change in sensitivity of the culture to metsulfuron-methyl with time was similar to that observed with NA (section 8.3.2). A doubling of culture mass over this period may have accounted for this response. However, actively dividing cells may also have been more sensitive to AHAS inhibition (and the resultant depletion of branched chain amino acids) than expanding cells in the later stages of culture growth.

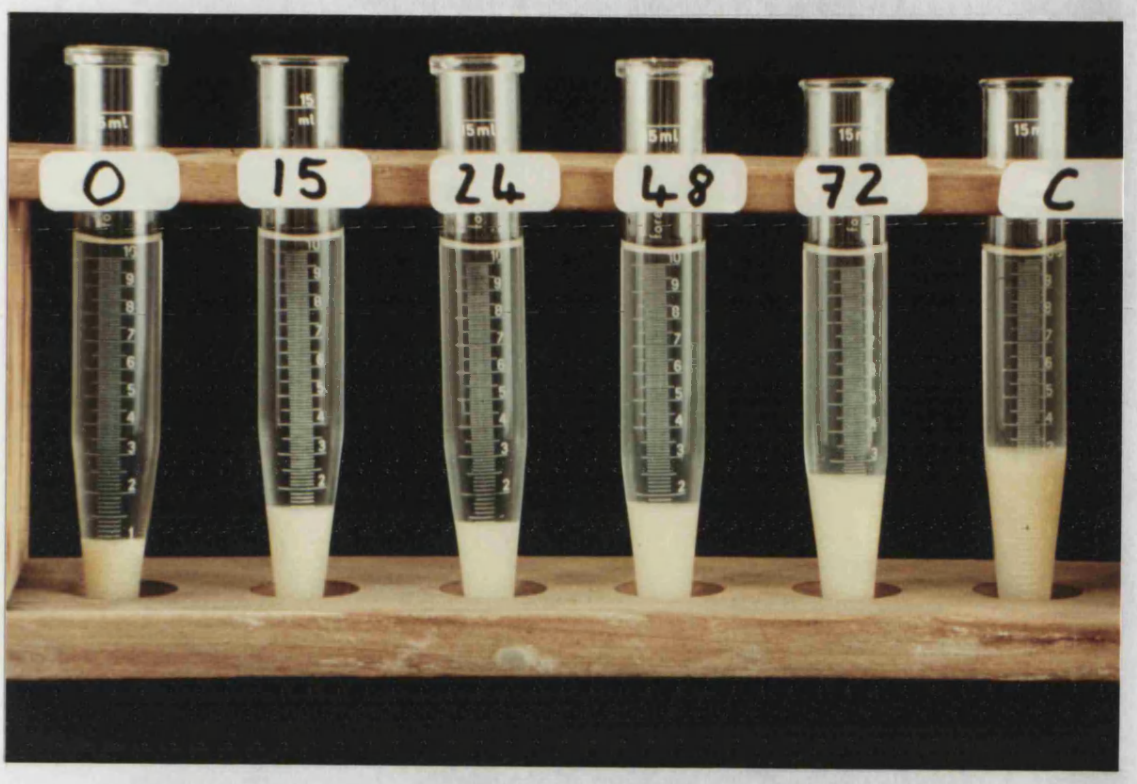
In order to ascertain the importance of culture mass vs. culture age on metsulfuron-methyl toxicity, a comparison of the effect of 1 ppm metsulfuron-methyl applied at 0 or 72 hours after subculture, on similar cell volumes, was made. By using X½ and X1 standard inoculum at subculture, X1 and X2 culture masses were obtained by day three. The effect of 1 ppm metsulfuron-methyl on x1 and x2 inoculum at subculture and X1 and X2 inoculum at day three was then compared.

Inhibition of similar culture masses was much more severe when metsulfuron-methyl was applied to cells at subculture, than 72 hours later (Figures 8.8, 8.9). Hence, the age of culture, and its stage of growth, and not only the mass of cells present, was important in determining metsulfuron-methyl toxicity.

FDA/Evans Blue staining of metsulfuron-methyl treated cultures gave no indication of a change in cell viability with herbicide action. Cultures treated with 1 ppm metsulfuron-methyl at day 0, which exhibited almost total inhibition of growth, had the same staining

Plate 8.10 Effect of time of application of 1 ppm metsulfuron-methyl on BMS settled cell volume at 7 days.

Plate 8.11 Settled cell volumes of control cultures for metsulfuron-methyl experiment at 7 days.


0
15
24
48
72
C

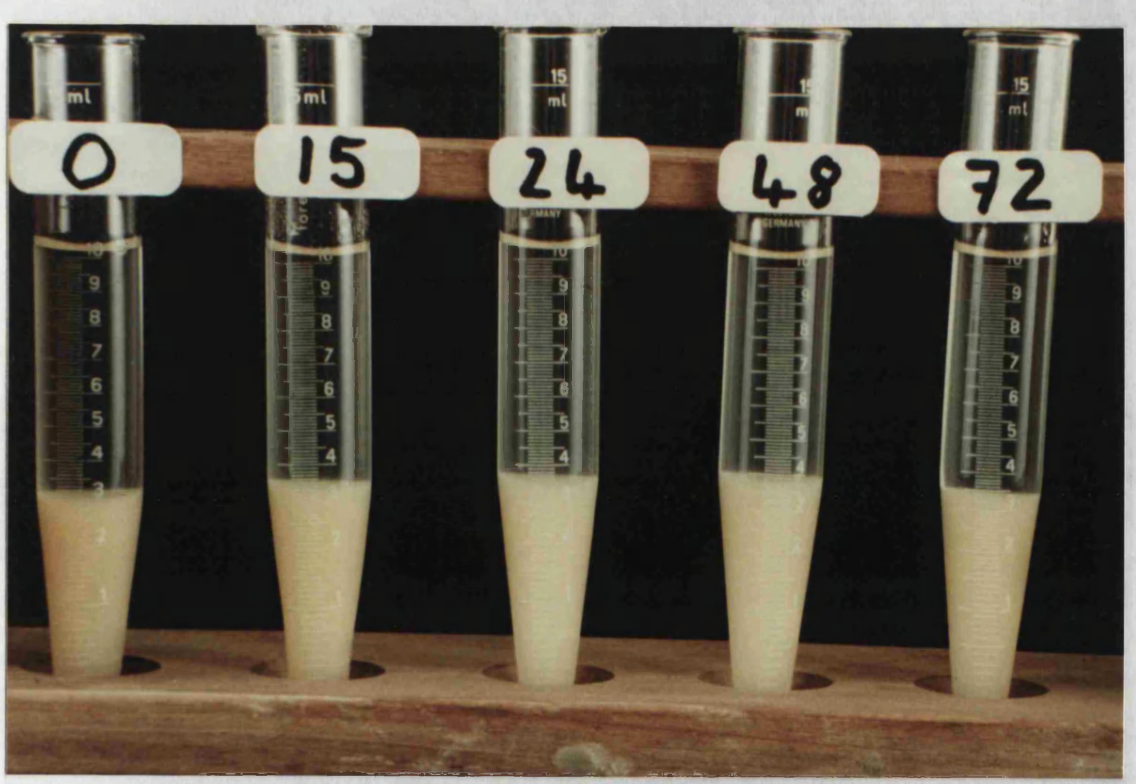
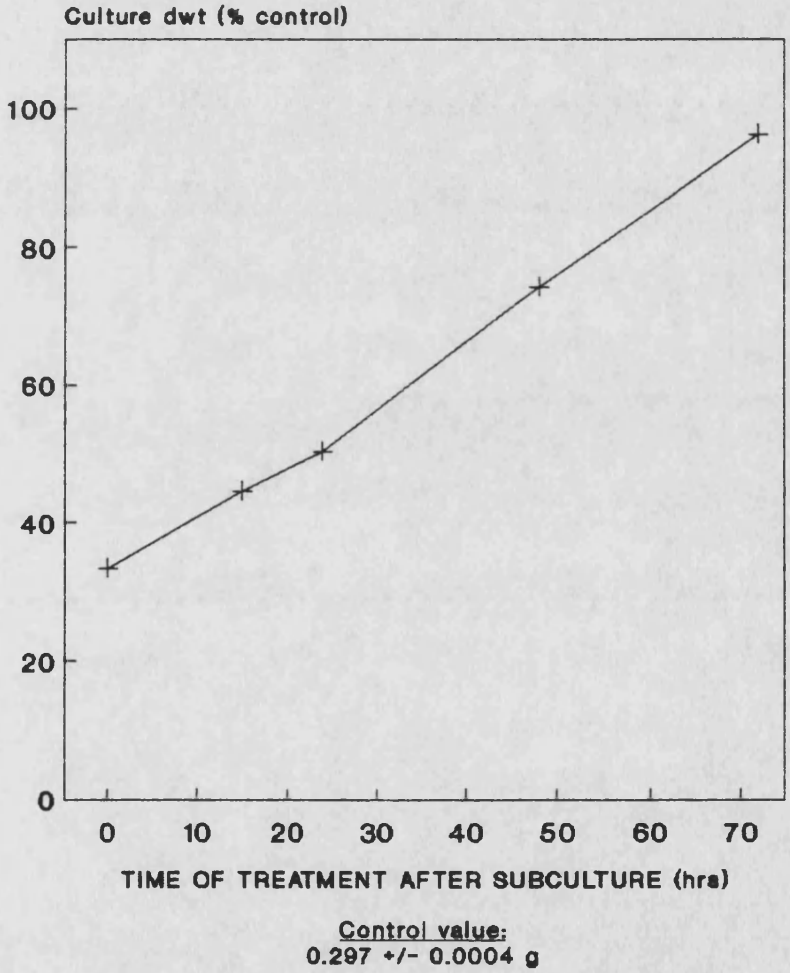
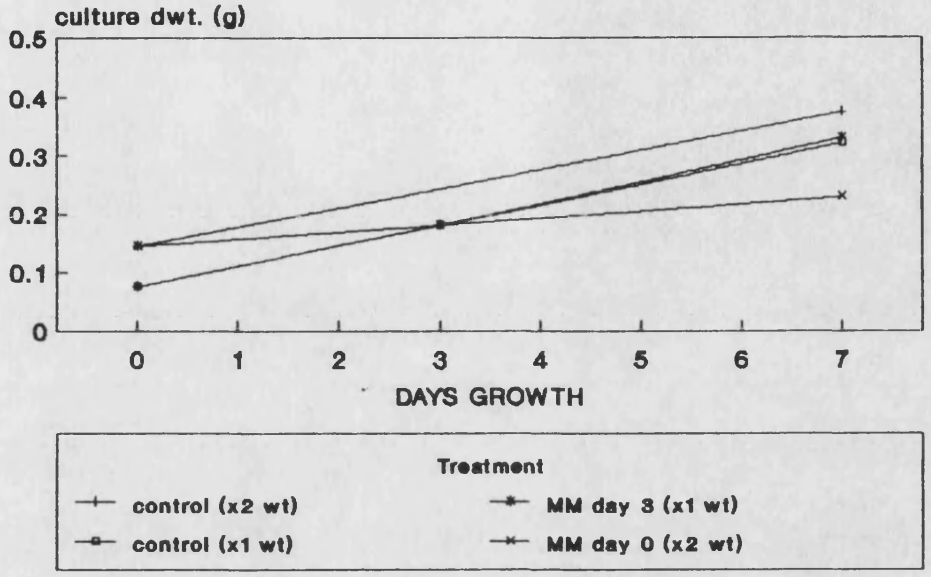

0
15
24
48
72

Fig. 8.7 Effect of time of 1 ppm metsulfuron-methyl treatment on BMS culture growth (dry weight + 7 days)



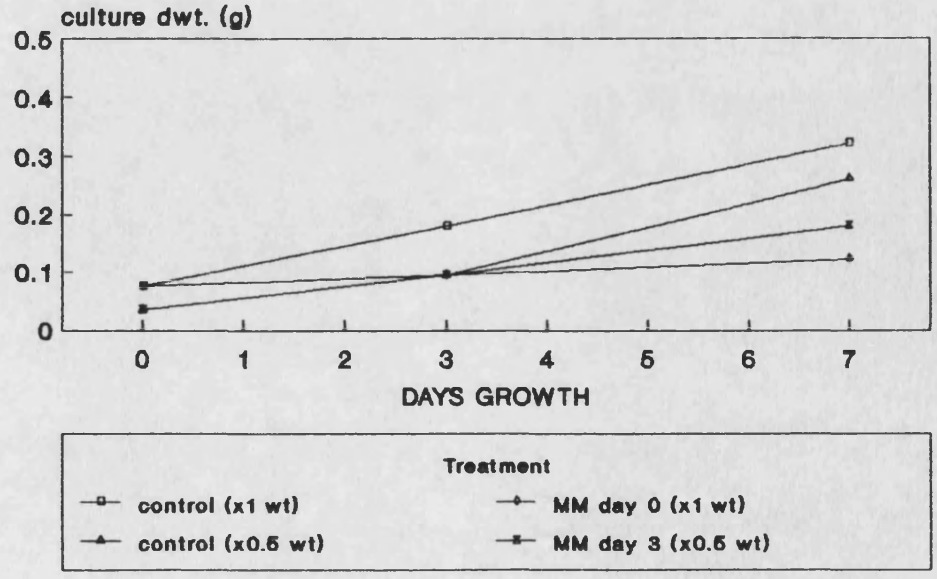
Control value:
0.297 +/- 0.0004 g

Fig. 8.8 Effect of cell culture weight and time of application of metsulfuron-methyl on inhibition of BMS growth



MM = metsulfuron-methyl

Fig. 8.9 Effect of cell culture weight and time of application of metsulfuron-methyl on inhibition of BMS growth



MM = metsulfuron-methyl

pattern as untreated cultures.

8.3.4 Interactive Effect of NA and Metsulfuron-methyl upon the Growth of BMS Cell Cultures

Whole plant investigations reported earlier in this thesis (section 3.3) indicated that NA could partially overcome growth inhibition of Z. mays L. plants by the sulfonylurea herbicide chlorsulfuron. Richardson, West and White (1984) reported a similar response to metsulfuron-methyl with NA in maize.

In looking for a possible antagonism of metsulfuron-methyl induced growth inhibition of BMS cultures by NA, a pretreatment period of one day with the safener was used. This would relate to whole plant treatments where the plant is exposed to NA as a seed dressing, before exposure to the herbicide. It would also allow biochemical changes to occur in the cells, such as enhancement of MFO activity, prior to herbicide treatment. Sweetser (1985) reported that NA treatment reduced the half-life of metsulfuron-methyl in maize leaves by enhancing MFO activity. Canivenc et al. (1988) reported an enhancement of chlortoluron metabolism in NA treated wheat cells with pretreatment, but not with simultaneous treatments at the same time.

NA was applied at the time of subculture at a concentration of 5×10^{-6} M. This concentration was chosen as it produced some inhibition of cell growth (30 and 20% of control growth by culture

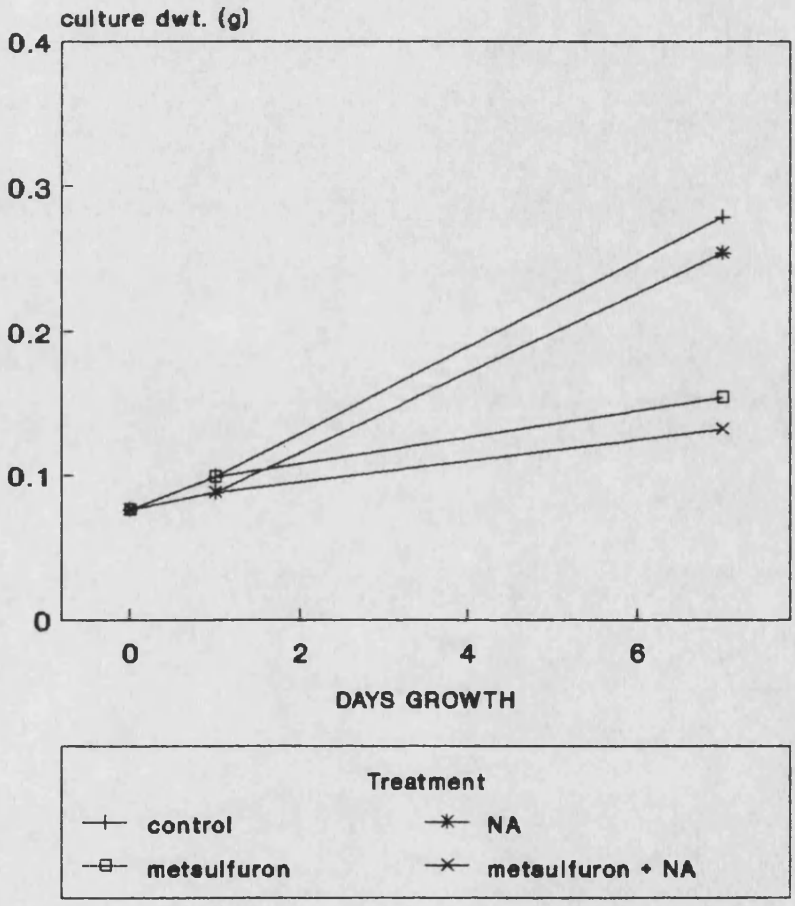
dry weight and settled cell volume respectively at day seven), but inhibition of growth would not be so severe as to mask the herbicide effect. A slight inhibition of growth was associated with NA action in whole plants (section 3.3). This response also indicated that NA was having an effect upon the cells, which might be associated with safening.

Metsulfuron-methyl was applied 24 hours after subculture at a concentration of 1 ppm. This level gave partial inhibition of culture growth (Fig. 8.7). Application later than this time would result in reduced herbicide effect, and hence little difference between growth of treated and untreated cultures where a safening response might be observed.

Figure 8.10 shows the effect of NA pretreatment at 5×10^{-6} M for one day on BMS growth with and without metsulfuron-methyl at 1 ppm. Control cultures showed a slight inhibition of growth with NA treatment. No antagonism of the metsulfuron-methyl effect upon BMS growth was seen in the presence of NA. A slight inhibition of growth above the metsulfuron effect was seen, similar to the NA effect in control cultures.

Figure 8.11 depicts the effect of 1.0 to 10 ppm metsulfuron-methyl applied three days after subculture on culture dry weight at day 10, and the interaction with 5×10^{-6} M NA and 0.5% v/v acetone applied at subculture. Metsulfuron-methyl in the absence of NA or acetone had no effect upon final culture dry weight at the

Fig. 8.10 Effect of 24 hr pretreatment with NA on BMS culture growth with 1 ppm metsulfuron-methyl (dwt +7 days)



NA concentration = 5x 10 -6 M

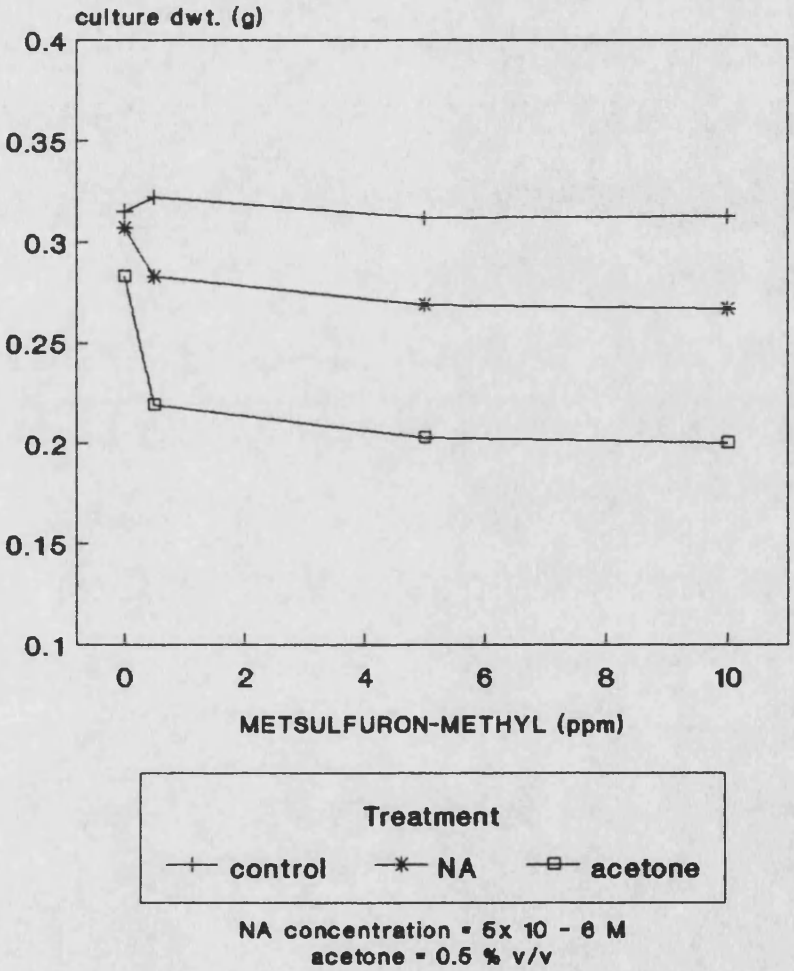
concentrations used. Addition of acetone caused a slight decrease in culture dry weight, and an increased herbicide effect, but this was not related to herbicide concentration. Addition of NA (in acetone) caused a further decrease in the final dry weight of control cultures and an enhanced herbicide effect. Inhibition was greater than with acetone and metsulfuron-methyl but was not dependent upon the metsulfuron-methyl concentration.

No antagonism of metsulfuron-methyl inhibition of BMS culture growth was seen with either one or three day pretreatments with NA. NA reduced culture dry weights to a similar extent in the presence of metsulfuron-methyl, as when applied alone. The enhanced herbicide effect seen with NA pretreatment for three days was probably due to BMS culture growth being partially inhibited compared to controls at day three, and hence being more sensitive to metsulfuron-methyl when it was applied (as depicted in Fig. 8.7). The enhanced herbicide effect in the presence of 0.5% acetone may also have been due to a slight inhibition of culture growth prior to herbicide treatment, or perhaps due to altered permeability of cell membranes to the herbicide (Davis, Wergin and Dusbabek, 1978).

8.3.5 The Effect of 2,4-D Concentration upon the Growth of BMS Cell Cultures: Interaction with NA and Metsulfuron-methyl

As 2,4-D has been found to alter the rate of metabolism of some herbicides in cell cultures, and antagonise the effect of other

Fig. 8.11 Effect of 72 hr pretreatment with NA +/- acetone on BMS culture growth with 1 ppm metsulfuron-methyl



NA concentration = 5x 10 - 6 M
acetone = 0.5 % v/v

herbicides on whole plants (section 8.1.2), possible interactions between 2,4-D and NA/metsulfuron-methyl were investigated.

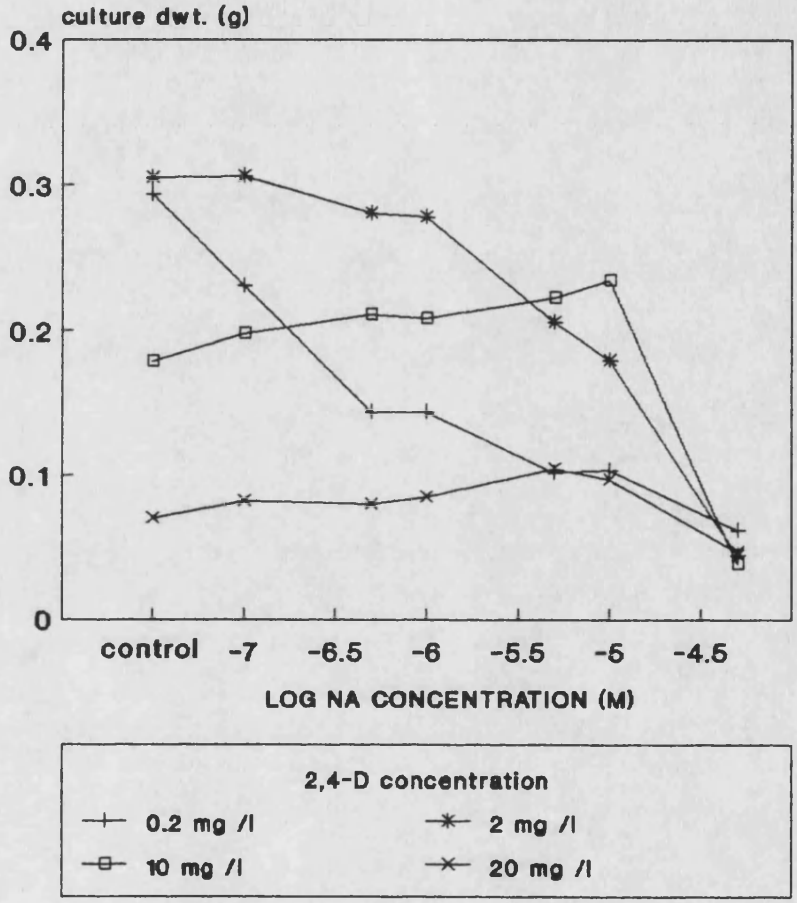
The NA dose response curve for BMS culture growth was compared at four different 2,4-D concentrations, 0.2, 2.0, 10 and 20 mg/l. Standard culture media for all previous experiments contained 2 mg/l 2,4-D.

There was an obvious effect of 2,4-D concentration upon BMS culture growth, and the response to NA treatments (Fig. 8.12). Growth of control cultures (NA free) was similar at 0.2 and 2.0 mg/l 2,4-D. However, both 10 and 20 mg/l 2,4-D resulted in decreased culture growth, with virtually no growth occurring at 20 mg/l. Cultures still showed a reduction in growth with NA concentration at 0.2 mg/l 2,4-D, however, inhibition was much more severe than was seen at 2 mg/l. At 10 mg/l 2,4-D the growth response altered, and cultures exhibited an increase in growth with NA concentration up to 10^{-5} M. There was also a marginal increase in culture growth with NA concentration at 20 mg/l 2,4-D.

It appeared from these results that 2,4-D antagonised the growth inhibition caused by NA at low 2,4-D concentrations, whereas NA partially counteracted the toxic effect of 2,4-D at higher 2,4-D concentrations.

The almost complete inhibition of BMS culture growth at 20 mg/l 2,4-D was contrary to the results of Cole and Owen (1987a) who

Fig. 8.12
Effect of 2,4-D on BMS culture growth with NA treatment (+7 days)



reported an increase in BMS culture growth at 20 mg/l 2,4-D, when compared to standard culture media of 5 mg/l. All NA treatments in this trial contained 0.5% v/v acetone, and this was found to enhance the toxic effect of 2,4-D (Figure 8.13). At 20 mg/l 2,4-D, culture growth was inhibited by 68% compared to 2 mg/l. However, at 20 mg plus 0.5% acetone, no culture growth was observed. The inhibitory effect of 20 mg/l 2,4-D in the absence of acetone was still marked compared to Cole and Owen (1987a). However, the two cultures were maintained at different 2,4-D levels, i.e. 2 and 5 mg/l, which may also have contributed to the greater sensitivity of the culture used in this trial, to 2,4-D. Somaclonal variation may also have accounted for differences of the two BMS cultures to 2,4-D. Cell cultures can become habituated to hormones, and lose their requirement for 2,4-D (Everett, 1981). Successful maintenance of this culture at lower 2,4-D levels may have reflected a degree of 2,4-D habituation by this culture.

The effect of 2,4-D concentration in culture media upon metsulfuron-methyl toxicity was also investigated. Figure 8.14 shows the effect of increasing 2,4-D from 0.2 to 10 mg/l on the activity of 1 ppm metsulfuron-methyl applied 36 hours after subculture.

Both control and metsulfuron-methyl culture weights decreased with increasing 2,4-D concentration. If the growth of metsulfuron-methyl treated cultures was expressed as a percentage growth of control cultures at each 2,4-D concentration, then the inhibitory effect of

Fig. 8.13
Effect of 0.5% acetone and 2,4-D on BMS culture growth (+7 days)

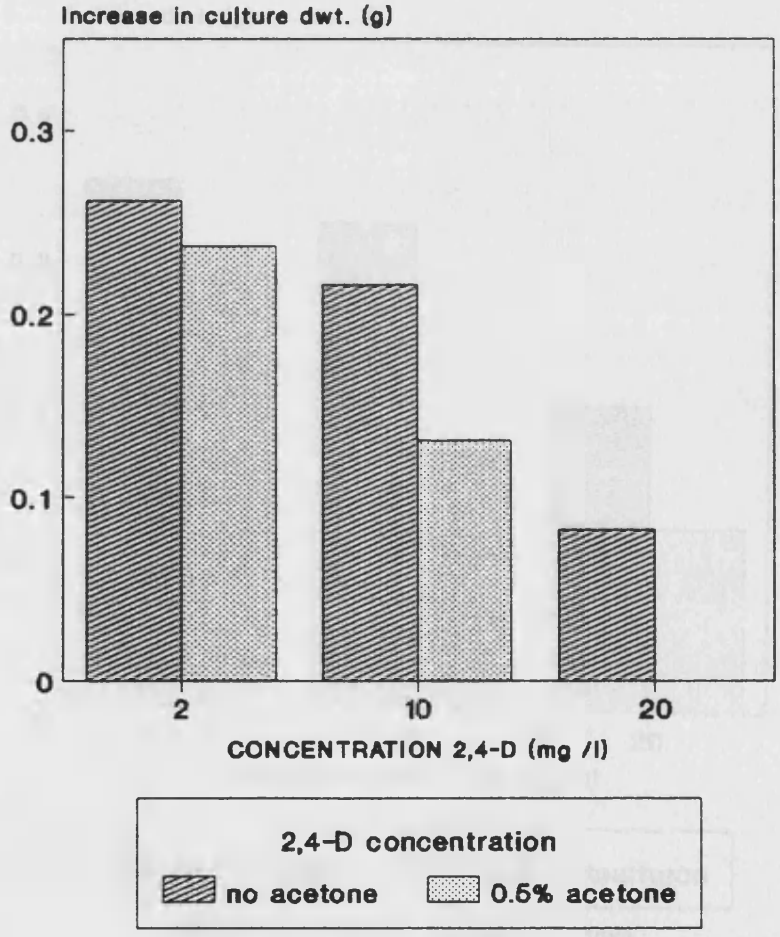
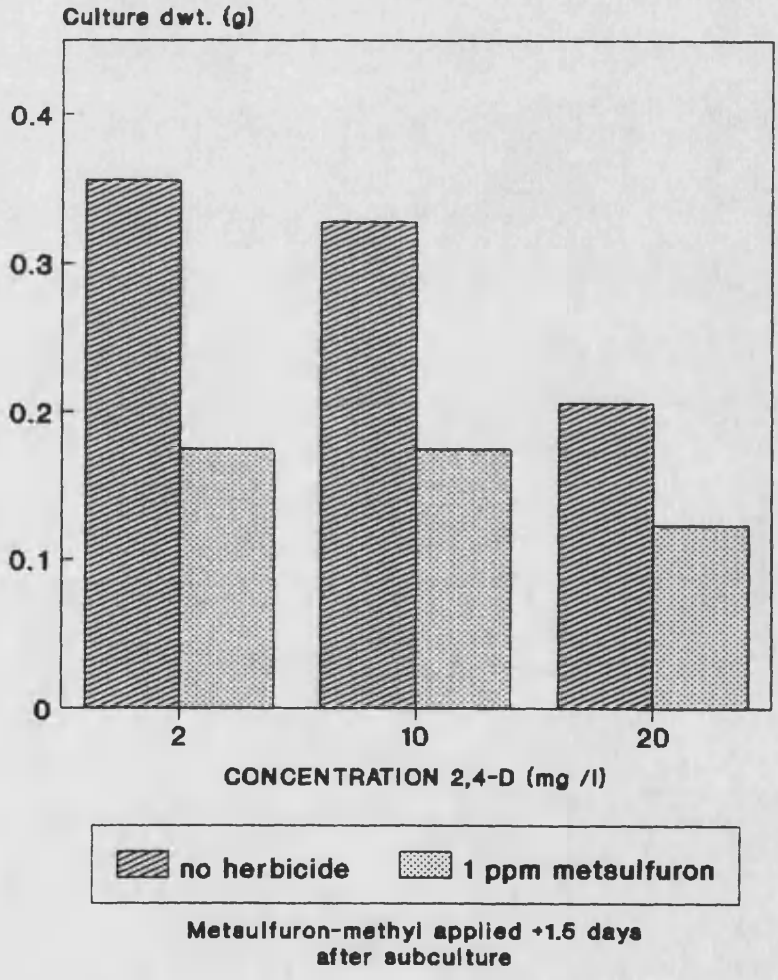


Fig. 8.14
Effect of 2,4-D +/- metsulfuron-methyl
on BMS culture growth (+7 days)



Metsulfuron-methyl applied +1.5 days
after subculture

Table 8.4 Effect of 2,4-D concentration on growth of BMS cultures with metsulfuron-methyl treatments

Concentration 2,4-D (mg/l)	Metsulfuron methyl (ppm)	Culture dry weights (g) day 1.5	day 7	Increase in dry weight (g)	Metsulfuron-methyl growth as % control
0.2	0	0.115	0.358	0.243	
	1	0.115	0.174	0.059	24.3
2.0	0	0.111	0.328	0.217	
	1	0.111	0.175	0.064	29.5
10	0	0.088	0.208	0.120	
	1	0.088	0.122	0.034	28.3

1 ppm metsulfuron-methyl was slightly greater at 0.2 mg 2,4-D than at 2 or 10 mg/l (Table 8.4). However, the difference did not indicate a clear antagonistic interaction between 2,4-D and metsulfuron-methyl.

8.3.6 Some Comments on the Effect of Acetone on Cell Culture Growth

The low water solubility of naphthalic anhydride, 2 ppm at 25°C (approx 10^{-5} M), necessitated the use of an organic solvent to dissolve it prior to addition to cell cultures. Davis, Wergin and Dusbabek (1978) reported that both the growth and ultrastructure of cells in culture were affected by a range of organic solvents. Acetone was less toxic than some solvents, including ethanol, and had a relatively low toxicity at concentrations of 0.5% v/v and below. However, concentrations of 1% v/v acetone and above caused changes in starch reserves and produced membrane abnormalities.

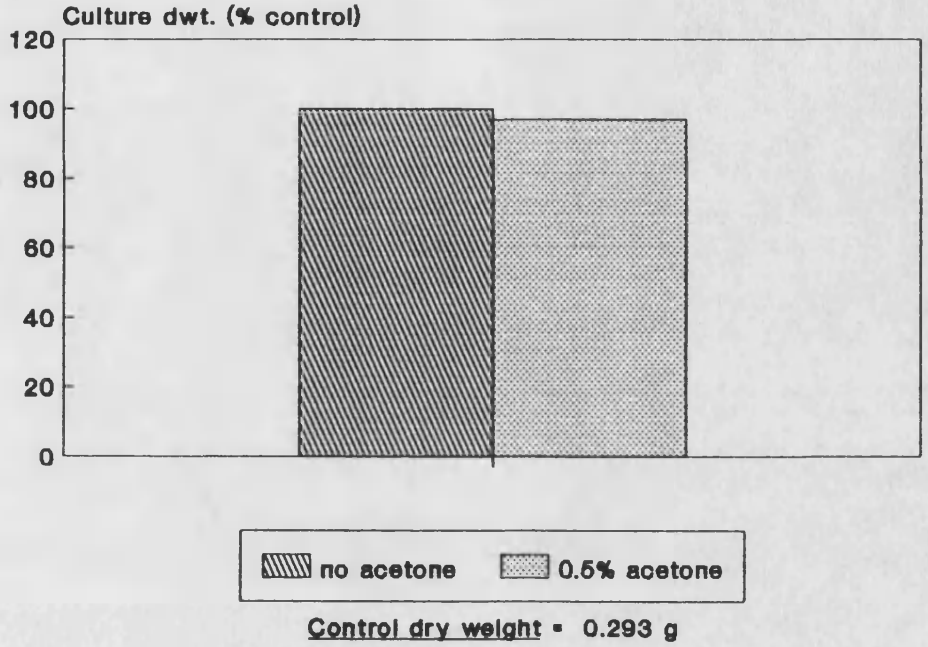
Of a range of solvents tested, solubility of NA was highest in acetone and ethanol. Because of the reported lower toxicity of acetone, it was chosen as the solvent, and used at a concentration of 0.5% v/v.

Initial trials with BMS cultures indicated that 0.5% v/v acetone caused less than a 5% reduction in culture growth over seven days (Fig. 8.15). However, results of experiments on metsulfuron-methyl and 2,4-D toxicity (Figs. 8.11 and 8.13) indicated that the

inclusion of acetone in culture media could alter the toxic effects of these compounds. Both metsulfuron-methyl and 2,4-D exhibited greater toxicity in the presence of acetone, with 2,4-D toxicity being enhanced greatly with increasing 2,4-D concentration (Fig. 8.13). Figure 8.16 shows that increasing acetone concentration from 0.1% to 0.75% reduced culture growth significantly at 10 mg/litre 2,4-D. 0.1% acetone had a greater effect on culture growth at 10 mg/litre 2,4-D than 0.5% at 2 mg/litre 2,4-D.

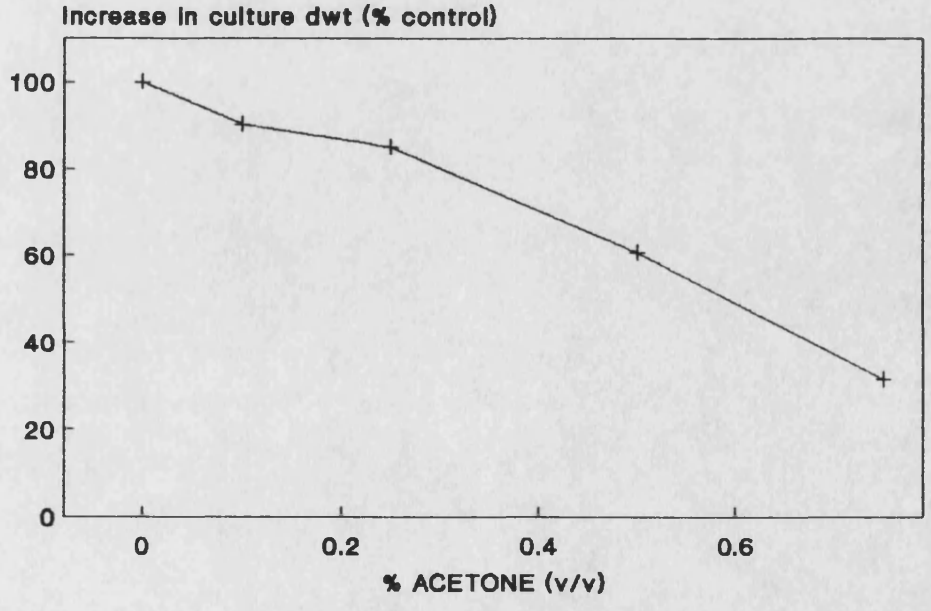
The results indicated that the effect of acetone on culture growth altered with the concentration of other phytotoxic compounds present. Furthermore, concentrations below 0.5% v/v had severe effects upon culture growth contrary to the results of Davis, Wergin and Dusbabek (1978). The results of NA inhibition of BMS culture growth (Figs. 8.3, 8.4, 8.5) may in part have reflected increasing toxicity of acetone with increasing NA concentration. However, no investigations were carried out at lower acetone concentrations to assess this hypothesis.

Fig. 8.15
Effect of 0.5% acetone on BMS culture growth at 2 mg/l 2,4-D (+7 days)



Control dry weight = 0.293 g

Fig. 8.16 Effect of acetone concentration on BMS culture growth at 10 mg/l 2,4-D (+7 days)



Control value = 0.218 g

8.4 DISCUSSION

No previous reports on the effect of NA on the growth of Zea mays cells in culture have been made, although stunting effects on whole plants have been observed (Hickey and Krueger, 1974; section 3.3 in this thesis).

NA reduced culture growth in a concentration dependent manner. Generation times of cultures were increased, but growth inhibition was not associated with cell death, except where NA inhibited growth totally.

The response of BMS cultures to NA was dependent upon the time of application after subculture. NA applied 72 hours after subculture was less inhibitory of subsequent growth, than when applied at subculture. Changes in the mass of cells present in the culture, and/or in the growth stage of the cells may have accounted for this. Ezra and Gressel (1982) also reported an effect of culture growth stage on the inhibitory effect of various compounds, with cultures being more sensitive during the initial lag-phase than during exponential growth.

Inhibition of culture growth by metsulfuron-methyl was also found to be dependent upon the time of application. Cultures were particularly sensitive to herbicide treatment at subculture. The change in culture sensitivity could not be accounted for by the mass of cells present at the time of treatment, thus the growth

stage of the cells was implicated as an important factor. As metsulfuron-methyl inhibits AHAS activity, and hence the biosynthesis of branched chain amino acids valine, leucine and isoleucine (Hawkes, Howard and Pontin, 1989), the results suggested that depletion of these amino acids had a more critical effect on culture growth during cell division, rather than during cell expansion. Ray (1980, 1982) found that chlorsulfuron inhibited cell division in plant roots, but not cell expansion. This is consistent with the effects of metsulfuron-methyl reported here.

The 2,4-D concentration of the cell culture media was found to have a significant effect upon the response of BMS cells to NA. Low levels of 2,4-D enhanced the inhibitory effect of NA on culture growth. However, high 2,4-D concentrations reversed the response of BMS cultures to NA, so that growth was enhanced with increased NA concentration.

Although 2,4-D has been reported to enhance the rate of metabolism of herbicides such as chlortoluron in cell cultures (Cole and Owen, 1987a; Canivenc et al., 1988), and antagonise the effect of several different herbicide groups in whole plants (Hatzios and Penner, 1985), 2,4-D concentration had no significant effect upon the toxicity of metsulfuron-methyl in the cell cultures.

NA had no antagonistic effect upon metsulfuron-methyl induced inhibition of culture growth. This was contrary to the report of Richardson, West and White (1984) where NA provided protection

against metsulfuron-methyl in Zea mays plants. However, as well as the difference between the two systems i.e. cell culture and whole plant material, Richardson, West and White (1984) used a different variety of maize (var. LG11). This may also have accounted for the difference in response between the two investigations.

The inhibitory effect of NA on BMS culture growth, and the interaction with 2,4-D may provide some clues as to the mechanism(s) involved in NA action. NA has been found to stimulate "auxin-like" growth responses in maize roots (Frear, Swanson and Mansager, 1987). Exogenous applications of the plant hormones abscisic acid (ABA), and gibberellic acid (GA_3) and the auxins indoleacetic acid (IAA) and phenylacetic acid (PAA) have been found to provide some protection to whole plants against growth inhibitor-type herbicides (Field and Caseley, 1987; Frear, Swanson and Mansager, 1987; Wilkinson, 1989). Hence, the growth response induced by NA, and interaction with 2,4-D may have been due to NA acting as a hormone/auxin with a specific receptor in the cell. Interaction with 2,4-D could thus be due to competition at a common site of action (Fig. 8.17a), or perhaps 2,4-D and NA acting at different sites, but having opposing effects on plant growth (Fig. 8.17b).

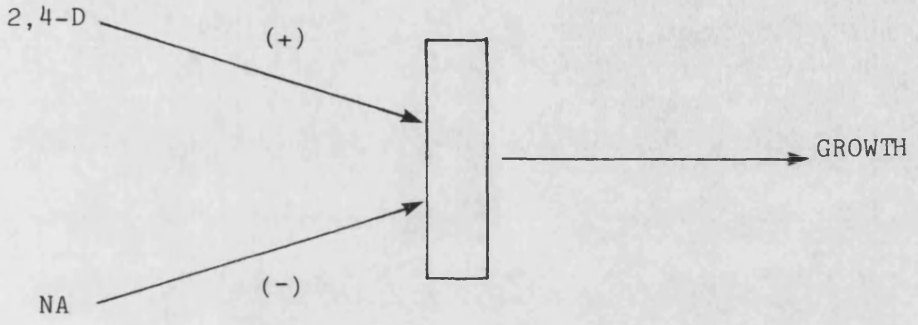
Alternatively, NA might alter plant and cell culture growth via effects on mixed function oxidase (MFO) activity. Sweetser (1985) suggested that the enhanced metabolism of sulfonylureas in maize following NA treatment was due to enhanced MFO activity.

Hydroxylation of 2,4-D and metabolism of GA_3 are believed to involve MFO action (Hatzios and Penner, 1982; Kömives and Dutka, 1989). Thus NA may alter plant growth by modulating levels of GA_3, and also reduce the growth of BMS cells in culture by enhancing the rate of 2,4-D metabolism (Fig. 8.17c). This might also account for the "safening effect" of NA against 2,4-D applied at toxic levels to cell cultures, as enhanced 2,4-D metabolism may have reduced the 2,4-D toxic effect.

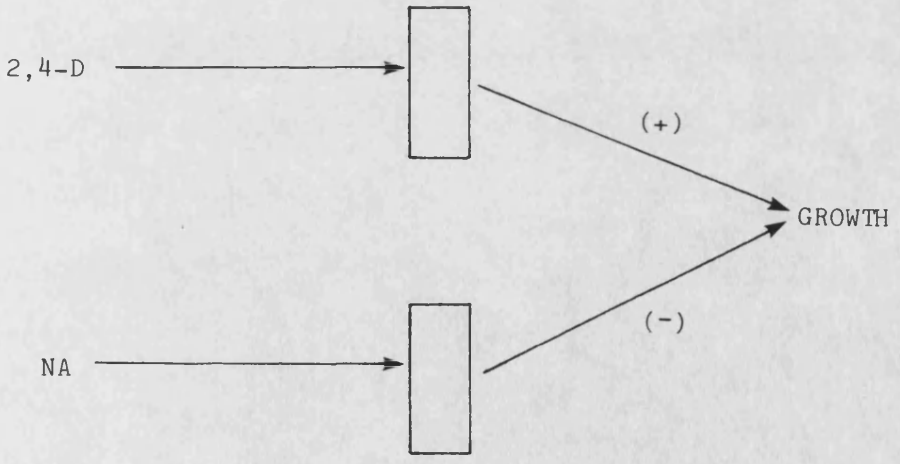
An effect on 2,4-D uptake into cultured cells may also have accounted for some of the antagonism betwen 2,4-D and NA on culture growth (Fig. 8.17d). Ezra, Krochmal and Gressel (1982) found competitive inhibition of EPTC uptake into maize cells with dichlormid and CDAA. Although NA had no effect on EPTC uptake (Ezra, Krochmal and Gressel, 1982), this does not preclude possible effects of NA upon 2,4-D uptake. Dichlormid has also been found to affect lipid metabolism in maize cells (Ezra and Gressel, 1982; Ezra, Gressel and Flowers, 1983). It is possible that NA may alter lipid metabolism and possibly also the lipid component and permeability of cell membranes to 2,4-D.

No information on the effect of NA on 2,4-D uptake and metabolism in the cells, or effects of 2,4-D on NA uptake was presented. The use of radiolabelled 2,4-D and NA may provide further information as to which, if any, of the mechanisms outlined were involved in the interaction between NA and 2,4-D.

Fig. 8.17 Suggested mechanisms for NA and 2,4-D interactive effect on BMS cell growth



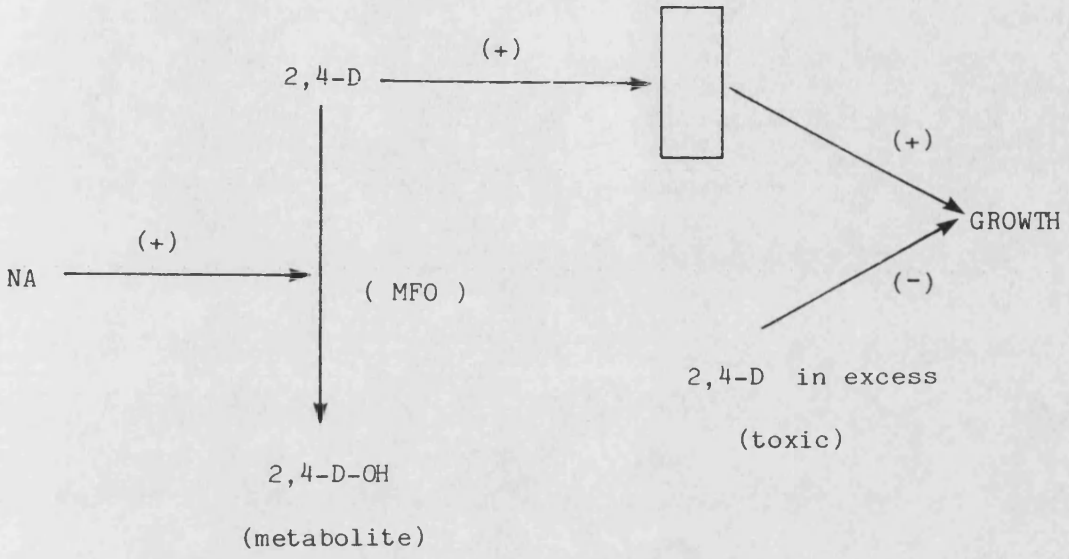
a) NA AND 2,4-D COMPETE AT THE SAME SITE OF ACTION



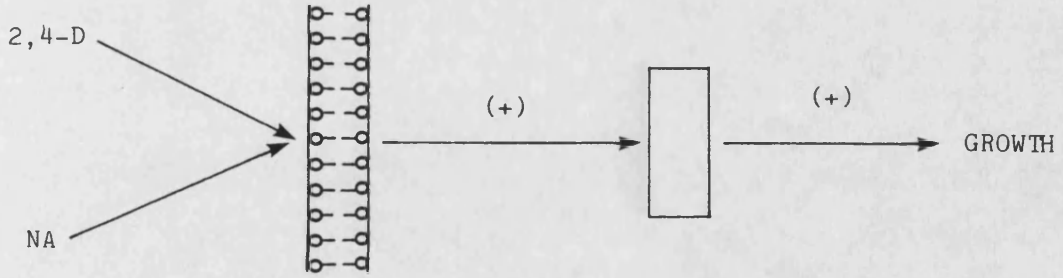
b) ACTION OF NA COUNTERACTS THE ACTION OF 2,4-D,

BUT CHEMICALS HAVE SEPARATE SITES OF ACTION.

Fig. 8.17 continued



c) NA ENHANCES THE METABOLISM OF 2,4-D



d) NA ALTERS 2,4-D UPTAKE, EITHER BY COMPETION AT THE SITE OF UPTAKE, OR ALTERATION OF CELL MEMBRANE PERMEABILITY.

Results of this investigation, and of previously reported work, indicate that there are several important factors which should be considered when using cell cultures to investigate herbicide action and safener interactions. These would also apply to the interpretation of results from this type of experiment.

The effect of NA and metsulfuron-methyl on subsequent culture growth was found to be dependent upon the growth stage at which they were applied. Inhibition of growth was much more severe when compounds were applied during the initial lag-phase and early exponential growth, than at later stages. Ezra and Gressel (1982) reported a similar response with other compounds. 2,4-D concentration in growth media was found to have significant effect upon the growth response with NA. Effects of 2,4-D on the rate of herbicide metabolism in cell cultures have also been reported (Canivenc et al., 1988; Cole and Owen, 1987a). Changes occurring during dedifferentiation, and with culture age due to somaclonal variation can lead to genetic and biochemical differences between plant and cell culture. Differences can also occur in cell cultures of the same species and variety, which are of different ages (Cole and Owen, 1988; Edwards and Owen, 1986 a, b, 1988; Larkin and Scowcroft, 1981; Lee and Phillips, 1987). Hence, comparisons between the results of safener/herbicide investigations using different cell cultures might lead to conflicting results. Similarly, relating cell culture responses to whole plants may also be difficult.

The use of organic solvents to introduce compounds into aqueous culture systems should also be treated with caution. The results of this investigation revealed that 0.5% v/v acetone was much more phytotoxic to cultures under certain conditions than was expected from the results of Davis, Wergin and Dusbabek (1978). Such effects should be considered if organic solvents are used in the production of herbicide dose response curves.

9. CONCLUDING DISCUSSION

The activity of herbicides in plants can be modified in the presence of a range of agrochemicals and other compounds (Hatzios and Penner, 1985). Synergistic combinations of herbicide/herbicide or herbicide/adjuvant are used in some formulations to enhance weed control. Conversely, the antagonistic effect of chemical safeners is used to improve the tolerance of some cereal crops to several types of herbicide (Hatzios, 1989a).

As with the development and use of herbicides, herbicide safeners and synergistic combinations have in general been screened for and then applied in agronomic situations, but the mechanisms by which they alter herbicide action have remained poorly understood. This has partly been due to a limited understanding of the mechanisms of action of many herbicides, and of herbicide metabolism in plants. Both of these factors are important if sites and mechanisms of herbicide x safener/synergist interaction are to be elucidated. Despite this, research into the mode of action of herbicide safeners has led to several possible mechanisms being proposed to explain their effects.

There is much evidence to indicate that an enhancement of herbicide metabolism is involved in the protective action of herbicide safeners. Several of the herbicides which are protected against are metabolised via glutathione conjugation; in particular the thiocarbamates and chloroacetanilides (Hatzios and Penner, 1982).

Most safeners which are active against these herbicides have been reported to enhance glutathione levels and/or glutathione-s-transferase activity in protected plants (Carringer, Rieck and Bush, 1978; Dutka and Kömives, 1987; Ezra et al., 1985; Fedtke, 1981; Gronwald et al., 1987; Kömives et al., 1985a; Lay, Hubbel and Casida, 1975; Rubin, Kirino and Casida, 1985; Stephenson, Ali and Ashton, 1983). Results in this present investigation confirmed that the dichloroacetamide safener dichlormid enhanced both glutathione levels and GST activity significantly in maize (Zea mays). However, several pieces of information suggest that other mechanisms may also be involved in safener protection against these herbicides.

Naphthalic anhydride (NA), which provides good protection to maize against thiocarbamate herbicides (Hatzios, 1983c) did not have any effect upon glutathione levels in this study. Kömives et al. (1985b) and Lay and Casida (1976) reported similar results. A doubling of GST activity in treated tissue towards CDNB was observed here and by other researchers (Fedtke, 1981; Kömives et al., 1985b; Mozer, Tiemeier and Jaworski, 1983). However, Lay and Casida (1976) found no enhancement of GST activity when EPTC sulfoxide was used as the assay substrate. GST has been reported to occur as at least two isoenzymes (Guddewar and Dauterman, 1979; Edwards and Owen, 1986b, 1988; Mozer, Tiemeier and Jaworski, 1983), and these exhibit different substrate specificities (Edwards and Owen, 1986b). Safener treatment has also been reported to enhance the activity of only one of two isoenzymes present in maize (Edwards and Owen, 1986b, 1988). Thus, although NA enhances GST

activity in maize towards CDNB, the results of Lay and Casida (1976) suggest that the isoenzyme induced is not active towards EPTC, and thus would not lead to an increase in the rate of GST-mediated metabolism of this herbicide. Hence, it would appear that enhancement of the glutathione/GST system might not account for the safening action of NA against the thiocarbamates.

Although Lay and Casida (1976) reported a good correlation between glutathione/GST enhancement and safening action for a range of compounds, several of the chemicals tested gave significant enhancement of glutathione and/or GST, but did not protect against herbicide damage. Conversely, some compounds which gave good protection produced virtually no change in glutathione or GST. Ezra and Gressel (1982) reported a rapid reversal of EPTC effects upon lipid biosynthesis in maize cells following dichlormid treatment. As this preceded any effect upon glutathione biosynthesis, this was suggested as the primary mechanism of safener action.

It is difficult to explain safener protection against other groups of herbicides which are not metabolised via glutathione conjugation, in terms of effects upon glutathione and GST. Although not investigated in this study, the mixed function oxidases (MFOs) which are believed to be involved in the detoxification of many herbicides (Hatzios and Penner, 1982), have been suggested as another possible site of safener action (Fedtke, 1987; Fedtke and Trebst, 1987; Sweetser, 1985). However, much of the evidence presented in this area is still unclear (Kömives and Dutka, 1989).

The involvement of MFOs in sulfonylurea metabolism (Sweetser, Schow and Hutchinson, 1982) and EPTC sulfoxidation (Leavitt and Penner, 1979; Dutka and Kömives, 1983) is not certain (Gronwald, 1989). Sweetser (1985) reported enhanced sulfonylurea metabolism in maize with a range of safeners, but these results have been disputed (Frear, Swanson and Mansager, 1987). The specificity of MFO inhibitors used to reverse the effect of safeners against herbicides (Hatzios, 1983,a,b) has also been questioned (Gronwald, 1989). Hence, further research into the effect of safeners upon MFO activity would be valuable in clarifying this situation, particularly if direct measurements of MFO activity were made. This would also help to substantiate the model proposed by Fedtke (1987) which attempted to describe herbicide, safener and synergist interactions in terms of effects upon the MFO system.

Antagonism of herbicides at their site of action has also been suggested as a mechanism of safener action (Hatzios, 1983c, 1989b). Since NA and dichlormid were found to provide partial protection to maize against the sulfonylurea herbicide chlorsulfuron in initial trials, the effects of these safeners upon acetohydroxyacid synthetase (AHAS), the target site of the sulfonylureas, was investigated.

NA significantly enhanced the level of AHAS in treated maize plants, whereas dichlormid had no effect. Neither safener enhanced the tolerance of AHAS in plants to chlorsulfuron, and NA had no *in vitro* effect upon inhibition of the enzyme by the herbicide. The

degree of AHAS enhancement by NA was not thought to be sufficient to account for the level of protection observed. Thus this was not believed to be a significant mechanism of protection against this herbicide. These results were similar to those of other researchers who found slight or no AHAS enhancement, and no effect of safeners upon AHAS sensitivity to chlorsulfuron, or inhibition *in vitro* (Barrett, 1989; Frear, Swanson and Mansager, 1987; Rubin and Casida, 1985).

Herbicide safeners have previously been reported to have an effect upon seed germination (Dill *et al.*, 1982; Davidson *et al.*, 1978) and early plant growth (Hickey and Krueger, 1974). Results presented here indicate that NA, oxabetrinil, flurazole and dichlormid all had a slight inhibitory effect upon maize growth when measured by leaf extension, but only flurazole had any significant effect upon plant fresh and dry weights at harvest. Flurazole was also the only safener to have any significant effect upon seed germination. Similar effects were observed with *Echinochloa crus-galli*.

The effect of safeners upon plant growth in the absence of herbicides may suggest either a "hormonal-like" characteristic of these compounds, or an effect upon plant hormone metabolism. It is interesting to note in this respect, that exogenous applications of plant hormones and auxins have been found to provide protection against several types of herbicide (Field and Caseley, 1987; Frear,

Swanson and Mansager, 1987; Wilkinson, 1989). NA has also been reported to produce "auxin-like" responses in maize (Frear, Swanson and Mansager, 1987).

2,4-D, one of the "hormonal type" herbicides, antagonises the toxic effect of several growth inhibitor type herbicides (Hatzios and Penner, 1985). It was also found to antagonise the inhibitory effect of NA upon maize cells grown in suspension culture, in this research. However, the reason for the antagonistic interaction between these two compounds was not ascertained. Further research in this area would help to establish if antagonism is due to a) alteration of herbicide/safener uptake into cells; b) effects of NA upon 2,4-D metabolism; or c) competitive antagonism at sites of action. Thus, there is some evidence to suggest a growth regulator-type mechanism for safener activity. This may relate to the observation that safening action is generally limited to growth-inhibitor type herbicides (Hatzios, 1989a). It would be interesting to establish what effect herbicide safeners have upon plant hormone and auxin levels. Similarly, it would also be useful to ascertain if exogenous applications of plant hormones have any effect upon herbicide metabolism, as has been observed with safeners. Cytokinins have been found to alter the rate of 2,4-D metabolism in soybean cell cultures (Montague et al., 1981).

It is probable that safeners induce a number of changes in plants, some or all of which contribute to the enhancement of herbicide tolerance. No one simple mechanism yet proposed is sufficient to

account for all safener x herbicide x species interactions.

Can herbicide activity and selectivity be improved by targeting specific enzymes involved in herbicide metabolism and/or mechanisms of herbicide activity?

Glutathione is involved in chloroplast protective mechanisms against superoxide damage (Dodge, 1989) one of the toxic species involved in paraquat toxicity (Dodge, 1983b). It is also involved in the metabolism of the thiocarbamates (Hatzios and Penner, 1982). Hence, modification of glutathione levels in plant tissue might alter the toxicity of these herbicides.

Results presented here showed that several chemicals which have previously been used to modulate glutathione biosynthesis in animal tissue, were effective as modulators of glutathione in plant tissue. 2-oxo-thiazolidine carboxylic acid (OTCA) and N-acetyl-L-cysteine (ALC) enhanced glutathione levels in pea leaf tissue, and buthionine-[S,R]-sulfoximine (BSO) reduced glutathione in pea leaf tissue and maize plants. However, none of the modulators altered the toxic effect of paraquat on pea leaf tissue, nor did BSO enhance the toxicity of EPTC in maize.

Despite the lack of success reported here, other researchers have managed to modify herbicide activity through a biorational approach. Gressel and Shaaltiel (1988) cite several examples of herbicide synergism where key metabolic processes have been

identified, and successfully modified, thus altering herbicide activity. Hilton and Pillai (1986) reported partial protection of maize against tridiphane (a herbicide metabolised by glutathione conjugation) following application of OTCA. The type of compounds used by these researchers will probably not be practical as commercial safeners or synergists, however, they may prove useful as tools used to increase our understanding of the mechanisms involved in determining herbicide activity.

Tissue cultures, which have been used to study both herbicide and safener effects upon isolated cells, may have some use as a system for screening for novel herbicide x safener/herbicide x synergist interactions. However, factors such as the stage of culture growth when compounds are added, the level of 2,4-D used in the culture media, and use of organic solvents to dissolve non-polar compounds in culture media, were all found to affect culture growth. Other factors such as organic nutrient levels and somaclonal variation occurring in tissue cultures may also alter the response of cells to herbicides, and their interaction with potential safeners/synergists. Hence, results of tissue culture investigations may not accurately reflect whole plant responses to combined chemical treatments.

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Appendix 1. Summary of anova for maize fresh weights (Fwt) and dry weights (Dwt) at 34 days for herbicide x safener treatments

Source of variation		F.pr. Herbicide level (1)		Safener level (2)		h. level x s. level (3)	
Herbicide	Safener	Fwt	Dwt	Fwt	Dwt	Fwt	Dwt
Met.	NA	0.952 (n.s.)	0.973 (n.s.)	0.486 (n.s.)	0.374 (n.s.)	0.775 (n.s.)	0.717 (n.s.)
	Ox.	0.398 (n.s.)	0.465 (n.s.)	0.117 (n.s.)	0.114 (n.s.)	0.840 (n.s.)	0.873 (n.s.)
	Fl.	0.430 (n.s.)	0.489 (n.s)	0.011 (*)	0.002 (**)	0.018 (*)	0.016 (*)
Atr.	NA	0.202 (n.s.)	0.095 (n.s.)	0.295 (n.s.)	0.228 (n.s.)	0.423 (n.s.)	0.384 (n.s.)
	Ox.	0.583 (n.s.)	0.595 (n.s.).	0.237 (n.s.)	0.188 (n.s.)	0.523 (n.s.)	0.664 (n.s.)
	Fl.	0.438 (n.s.)	0.421 (n.s.)	0.156 (n.s.)	0.129 (n.s.)	0.844 (n.s.)	0.846 (n.s.)
Chl.	NA	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)	0.059 (n.s.)	0.137 (n.s.)
	Ox.	0.001 (***)	0.001 (***)	0.026 (*)	0.015 (*)	0.001 (***)	0.003 (**)
	Fl.	0.001 (***)	0.001 (***)	0.559 (n.s.)	0.138 (n.s.)	0.032 (*)	0.002 (**)
Ima.	NA	0.001 (***)	0.001 (***)	0.343 (n.s.)	0.646 (n.s.)	0.070 (n.s.)	0.109 (n.s.)
	Ox.	0.001 (***)	0.001 (***)	0.309 (n.s.)	0.188 (n.s.)	0.913 (n.s.)	0.916 (n.s.)
	Fl.	0.001 (***)	0.001 (***)	0.492 (n.s.)	0.256 (n.s.)	0.880 (n.s.)	0.900 (n.s.)

(1) 7 d.f.	n.s.	$p > 0.05$
(2) 7 d.f.	*	$0.01 < p \leq 0.05$
(3) 1 d.f.	**	$0.001 < p \leq 0.01$
	***	$p \leq 0.001$

Appendix 2. Summary of anova for the length of the third leaf of maize with herbicide x safener treatments +14 days after sowing.

		Herbicide level (1)		Safener level (2)		h. level x s. level (3)	
Herbicide	Safener	F.pr	Sig(4)	F.pr	Sig	F.pr	Sig.
Met.	NA	0.871	n.s.	0.002	**	0.598	n.s.
	Ox.	0.699	n.s	0.034	*	0.633	n.s.
	Fl.	0.150	n.s.	0.033	*	0.254	n.s.
Atr.	NA	0.458	n.s.	0.101	n.s.	0.294	n.s.
	Ox.	0.535	n.s.	0.019	*	0.767	n.s.
	Fl.	0.479	n.s.	0.259	n.s.	0.410	n.s.
Chl.	NA	0.001	***	0.001	***	0.013	*
	Ox.	0.001	***	0.046	*	0.078	n.s.
	Fl.	0.001	***	0.430	n.s.	0.052	n.s.
Ima.	NA	0.001	***	0.103	n.s.	0.270	n.s.
	Ox.	0.001	***	0.001	***	0.006	**
	Fl.	0.001	***	0.002	**	0.364	n.s.

(1) 3 d.f.
(2) 7 d.f.
(3) 9 d.f.

(4) Significance:

n.s.	$p > 0.05$
*	$0.01 < p \leq 0.05$
**	$0.001 < p \leq 0.01$
***	$p \leq 0.001$

Appendix 2 continued. Summary of anova for the length of the third leaf of maize with herbicide x safener treatments + 18 days after sowing.

		Herbicide level (1)		Safener level (2)		h. level x s. level (3)	
Herbicide	Safener	F.pr	Sig(4)	F.pr	Sig.	F.pr	Sig.
Met.	NA	0.282	n.s.	0.007	**	0.845	n.s.
	Ox.	0.656	n.s	0.075	n.s.	0.799	n.s.
	Fl.	0.268	n.s.	0.078	n.s.	0.028	*
Atr.	NA	0.353	n.s.	0.095	n.s.	0.461	n.s.
	Ox.	0.050	(*)	0.001	***	0.293	n.s.
	Fl.	0.908	n.s.	0.528	n.s.	0.874	n.s.
Chl.	NA	0.001	***	0.001	***	0.003	**
	Ox.	0.001	***	0.006	**	0.031	*
	Fl.	0.001	***	0.364	n.s.	0.033	*
Ima.	NA	0.001	***	0.699	n.s.	0.439	n.s.
	Ox.	0.001	***	0.046	*	0.563	n.s.
	Fl.	0.001	***	0.065	n.s.	0.482	n.s.

(1) 3 d.f.
(2) 3 d.f.
(3) 9 d.f.

(4) Significance:

n.s.	$p > 0.05$
*	$0.01 < p \leqslant 0.05$
**	$0.001 < p \leqslant 0.01$
***	$p \leqslant 0.001$

Appendix 2 continued. Summary of anova for the length of the third leaf of maize with herbicide x safener treatments +24 days after sowing

		Herbicide level (1)		Safener level (2)		h. level x s. level (3)	
Herbicide	Safener	F.pr	Sig(4)	F.pr	Sig	F.pr	Sig.
Met.	NA	0.189	n.s.	0.012	*	0.802	n.s.
	Ox.	0.893	n.s	0.278	n.s.	0.668	n.s.
	Fl.	0.255	n.s.	0.223	n.s.	0.036	*
Atr.	NA	0.117	n.s.	0.004	**	0.111	n.s.
	Ox.	0.118	n.s.	0.001	***	0.531	n.s.
	Fl.	0.851	n.s.	0.695	n.s.	0.984	n.s.
Chl.	NA	0.001	***	0.001	***	0.004	**
	Ox.	0.001	***	0.007	**	0.073	n.s.
	Fl.	0.001	***	0.020	*	0.122	n.s.
Ima.	NA	0.001	***	0.479	n.s.	0.483	n.s.
	Ox.	0.001	***	0.205	n.s.	0.805	n.s.
	Fl.	0.001	***	0.585	n.s.	0.880	n.s.

(1) 3 d.f.
(2) 3 d.f.
(3) 9 d.f.

(4) Significance:

n.s.	$p > 0.05$
*	$0.01 < p \leq 0.05$
**	$0.001 < p \leq 0.01$
***	$p \leq 0.001$

Appendix 3. Effect of safener application level on the mean length of maize third leaf, for "unsafened" herbicide treatments.

(i) 14 days after sowing

Herbicide	Safener	Mean leaf length (cm) (3) Safener level (2) 0	1	2	3	anova (1) (significance)
Met.	NA	12.39	9.79	10.01	8.84	**
Atr.		10.32	9.67	8.23	8.69	n.s.
Ima.		9.52	7.50	7.76	7.74	n.s.
Met.	Ox.	11.11	10.16	10.57	7.75	*
Atr.		11.84	10.12	8.94	8.53	**
Ima.		11.18	7.75	7.56	6.74	***
Met.	Fl.	10.06	7.95	8.53	5.39	*
Atr.		8.29	8.13	9.37	7.03	n.s.
Ima.		9.59	7.22	7.10	4.68	**

(1) Significance levels:

n.s.	$p > 0.05$
*	$0.01 < p \leqslant 0.05$
**	$0.001 < p \leqslant 0.01$
***	$p \leqslant 0.001$

(2) Safener levels (by seed weight)

NA	0, 0.25, 0.5, 1.0%
Oxabetrinil	
Flurazole	0, 0.5, 1.0, 2.0%

(3) Average of 4 values.

Appendix 3. continued.

(ii) 18 days after sowing

Herbicide	Safener	Mean leaf length (cm) (3) Safener level (2) 0	1	2	3	anova (1) (significance)
Met.	NA	28.99	26.67	25.15	24.44	**
Atr.		26.17	24.83	21.69	23.18	n.s.
Ima.		18.02	18.30	16.39	17.44	n.s.
Met.	Ox.	26.96	27.39	26.88	22.74	n.s.
Atr.		28.83	24.79	25.67	22.79	***
Ima.		21.41	17.79	18.21	16.50	*
Met.	Fl.	23.69	23.49	23.80	18.44	n.s.
Atr.		24.42	22.60	24.71	22.14	n.s.
Ima.		19.34	16.54	16.46	14.31	n.s.

(1) Significance levels:

n.s.	$p > 0.05$
*	$0.01 < p \leq 0.05$
**	$0.001 < p \leq 0.01$
***	$p \leq 0.001$

(2) Safener levels (by seed weight)

NA	0, 0.25, 0.5, 1.0%
Oxabetrinil	
Flurazole	0, 0.5, 1.0, 2.0%

(3) Average of 4 values.

Appendix 3. continued.

(iii) 24 days after sowing

Herbicide	Safener	Mean leaf length (cm) (3) Safener level (2) 0	1	2	3	Anova (1) (significance)
Met.	NA	32.11	30.15	28.09	26.91	*
Atr.		30.06	28.21	24.11	25.76	**
Ima.		19.48	20.92	18.00	19.14	n.s.
Met.	Ox.	30.02	30.54	30.03	26.88	n.s.
Atr.		32.31	28.96	29.75	26.57	***
Ima.		23.23	20.24	20.51	18.43	n.s.
Met.	Fl.	27.36	27.96	25.70	24.20	n.s.
Atr.		29.26	26.79	27.56	27.06	n.s.
Ima.		21.08	19.59	18.45	18.43	n.s.

(1) Significance levels:

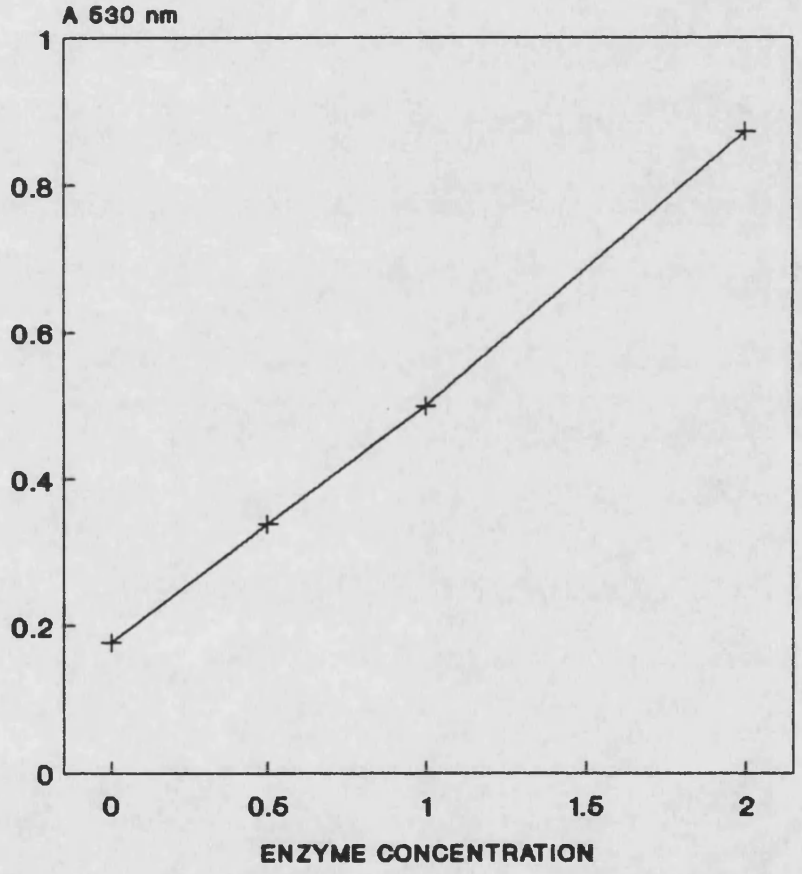
n.s.	$p > 0.05$
*	$0.01 < p \leq 0.05$
**	$0.001 < p \leq 0.01$
***	$p \leq 0.001$

(2) Safener levels (by seed weight)

NA	0, 0.25, 0.5, 1.0%
Oxabetrinil	
Flurazole	0, 0.5, 1.0, 2.0%

(3) Average of 4 values.

Appendix 4.
AHAS assay. Response of A 530 to enzyme concentration



1 enzyme concentration = 125 µl extract. (0.75 g leaf material ground in 6.5 ml buffer and desalted. Assay period 60 min

BIOCHEMICAL ASPECTS OF SAFENER ACTION: EFFECTS ON GLUTATHIONE, GLUTATHIONE -S- TRANSFERASE AND ACETOHYDROXY ACID SYNTHETASE IN MAIZE

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ABSTRACT

The chemical safener N,N-diallyl -2,2 -dichloroacetamide (DDCA) increased the glutathione content of root tissue, and glutathione-S-transferase activity in the root and shoot tissues of the treated maize (Zea mays) plants. Napthalic anhydride (NA) also enhanced glutathione-S-transferase activity in root and shoot, but had no effect upon glutathione content.

Both NA and DDCA enhanced the activity of acetohydroxy acid synthetase (AHAS), the target site of sulfonylureas, in treated plants. However, AHAS extracted from safened plants was more sensitive to chlorsulfuron inhibition. NA in vitro did not alter AHAS inhibition by chlorsulfuron.

INTRODUCTION

The herbicide safeners napthalic anhydride (NA) and N,N-diallyl -2,2 -dichloroacetamide (DDCA) enhance the tolerance of maize (Zea mays) to thiocarbamate herbicides, and to a lesser extent, the sulfonylureas (Hatzios 1983 Parker 1983).

Although much research has been directed towards their mode of action, the biochemical basis for the protective action of these safeners in maize is still not clearly understood.

DDCA enhances the level of glutathione-S-transferases (G-S-T) and reduced glutathione (GSH) in treated tissue (Lay and Casida 1976, Mozer et al 1983, Komives et al 1985, Lay and Niland 1985), and hence the rate of thiocarbamate detoxification via GSH conjugation of their sulfoxides. G-S-T isoenzymes have been identified in maize (Guddewar and Dauterman 1979, Mozer et al 1983). These have different herbicidal specificities, and the induction of isoenzymes particular to certain herbicide molecules, may account for the specificity of safener action. (Edwards and Owen 1986).

Sweetser (1985) reported an enhanced rate of sulfonylurea metabolism in NA/DDCA treated tissue, which was believed to be associated with an induction of the cytochrome P-450 mixed function oxidase system (Fedtke 1987).

Because of the association between safener action and the induction/enhancement of enzyme systems in treated tisue, it seemed appropriate to investigate the effect of these safeners upon acetohydroxy acid synthetase (AHAS) activity, the target site of the sulfonylurea

herbicides (Ray 1984). If either the total amount of AHAS is altered considerably, or isoenzymes are induced with decreased sulfonylurea sensitivity, then this might in part explain the safening action reported by Parker (1983).

MATERIALS AND METHODS

Plant Material

Seeds of maize (var LG 11) were washed in water to remove fungicide dressing, before applying either NA (97% w/w a.i.) or DDCA (20% w/w a.i.) at rates of 0, 0.25, 0.5 and 1.0% by seed weight.

Three seeds were sown per 9 cm pot in Vermiculite and watered thoroughly with 50% Hoaglands solution. Pots were placed in a light cabinet (16 hours light, 390 μ E M^{-2} S^{-1} 27/19^{o}C), and watered every two days with 50 ml of nutrient solution.

GSH and G-S-T assays

Both assays were carried out using root and shoot tissue of 5 day old plants. (2 leaf stage).

GSH was assayed spectrophotometrically by following the reduction of 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) by GSH in the presence of NADPH and glutathione reductase (Low et al 1983, Smith et al 1984).

G-S-T activity was measured spectrophotometrically using 1-chloro-2,4, dinitrobenzene (CDNB) as a substrate. (Mozer et al 1983). Both assays were carried out at 25^{o}C.

AHAS extraction and assay

Root and leaf tissue was used from 7 day old plants (3 leaf stage). The methods used were based upon those of Chaleff and Mauvais (1984) as modified by R. Wallsgrove (personal communication).

(i) Leaf Extraction

Approximately 0.8g of the youngest leaves of plants was homogenised on ice in 5.5 ml of extraction buffer containing; 50 mM KH_2PO_4, 5 mM $MgSO_4$, 10 mM pyruvate, 0.5 mM cocarboxylase, 10 μ M flavin adenine dinucleotide (FAD), 1 mM L-leucine, 1 mM L-valine, 10% ethanediol (v/v), 0.05% Triton X 100, at pH 7.5, with 50% w/w polyvinyl polypyrrolidone (PVPP). The crude extract was strained through 4 layers of muslin, and centrifuged at 11,600g for 8 minutes. 2ml was desalted on a Sephadex G 25 column equilibrated with resuspension buffer containing 50 mM KH_2PO_4 pH 7.5, 5mm $MgSO_4$, 10 mM pyruvate, 30% ethanediol.

(ii) Root Extraction

Approximately 5g of root tissue was ground in a pestle and mortar in 10 ml extraction buffer with 0.5g PVPP and a small amount of washed sand. The resulting brei was strained through 4 layers of muslin, and spun at 4000g for 5 minutes. AHAS was precipated from the supernatant solution using

$(NH_4)_2SO_4$ between 25 and 50% saturation. The pellet collected by centrifuging for 20 minutes at 10,000g was resuspended in 2ml of resuspension buffer and desalted as above.

Assay

In a total of 650 µ l, the assay contained; 50 mM KH_2PO_4 pH 7.5, 50 mM pyruvate, 10 mM $MgSO_4$, 250 µM cocarboxylase, 20 µ M FAD, 125 µ l enzyme, and as required, 125 µ l chlorsulfuron. Samples were incubated at 30°C for 1 hour, when the reaction was stopped by the addition of 125 µ l of 3 M H_2SO_4. After incubation at 60°C for 15 minutes, the acetoin content of samples was determined by the sequential addition of 125 µ l 20% NaOH, 187 µ l 0.5% Creatine, 187 µl 5% ∝napthol. After 1 hour the samples were centrifuged and absorption at 530 nm recorded.

AHAS activity was expressed as a percentage values by comparison with the absorption values of treatments with samples incubated without chlorsulfuron. Where NA was used in vitro, it was first dissolved in acetone, before diluting in the phospate assay buffer. Final acetone concentration in the assay mixture was 0.25% v/v.

Protein assay

The protein content of enzyme extracts was determined using the method of Bradford (1976).

RESULTS

Treatment of maize seeds with NA and DDCA enhanced the activity of G-S-T in root and shoot tissue in a dose dependent manner (figure 1). The rsponse to DDCA treatments was greater than with equivalent NA treatments. Root and shoot tissue showed similar G-S-T enhancement with each safener (two to three fold at 1%).

No significant GSH response was found with DDCA in shoot tissue, or with NA treatment in root or shoot. However, DDCA did enhance the GSH content of root tissue in a dose dependent manner. (Figure 2).

At an application rate of 0.5% by seed weight, NA gave an increase in extractable AHAS activity of 1.25 to 1.5 fold, in both root and third leaf after 7 days. (Table 1). The response to DDCA was less however, with an increase of between 1.0 to 1.3 fold. These responses were less than those shown for G-S-T at the 0.5% application rate i.e. approximately a two fold increase in activity for both safeners.

To investigate the possible induction of a chlorsulfuron resistant isoenzyme of AHAS with safener treatment, inhibition curves were plotted for AHAS extracted from safened and unsafened tissue. Figures 3 a and b reveal that both NA and DDCA pretreatments at 0.5% decreased the activity of the extracted AHAS over a range of chlorsulfuron treatments, when compared with non-safened plants.

The addition of NA in vitro (10^{-5} -10^{-8} M), to AHAS extracted from unsafened plants, resulted in a 10 - 15% decrease in activity as compared with an NA free control (Figure 4). A similar result was found with the same NA concentrations in the presence of 10 ppb chlorsulfuron.

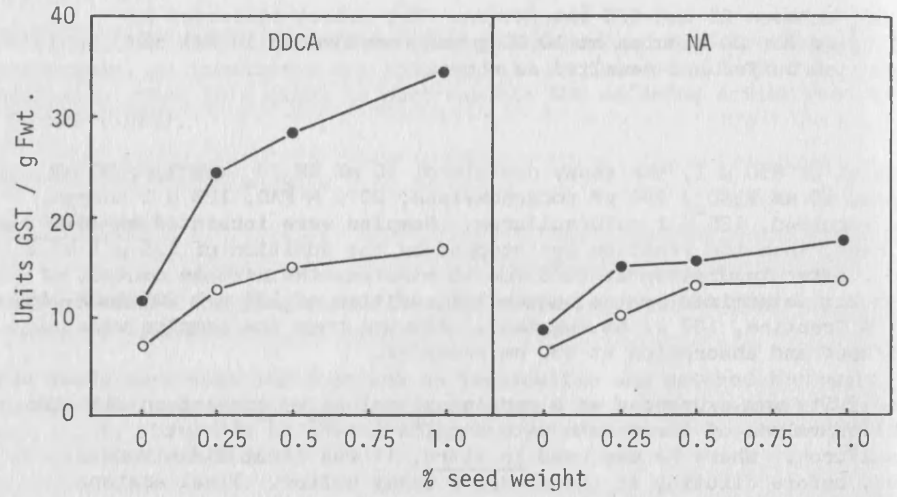

Fig. 1. Effect of DDCA and NA upon G-S-T activity in root (●——●) and shoot (○——○) tissue 5 days after treatment.

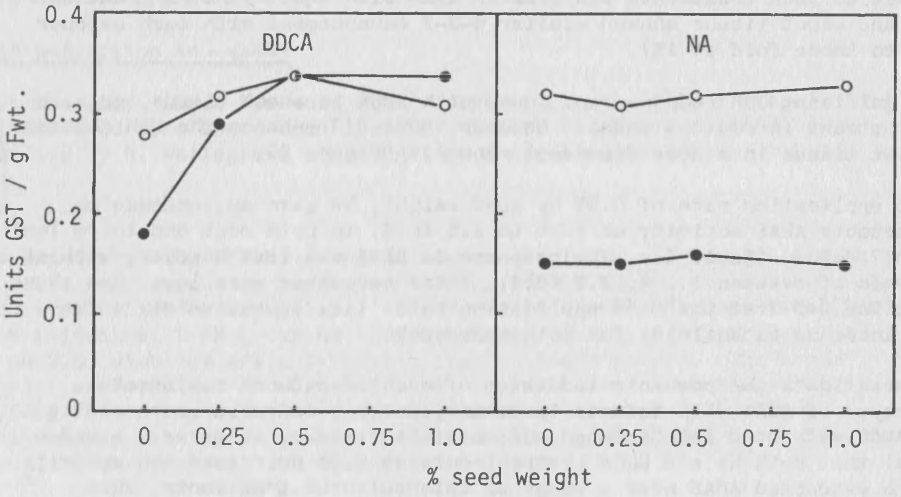

Fig. 2. Effect of DDCA and NA upon GSH levels in root (●——●) and shoot (○——○) tissue 5 days after treatment.

TABLE 1.

Effect of DDCA / NA as seed dressing at 0.5% by seed weight on extractable AHAS activity 7 days after treatment.

Treatment	Leaf tissue		Root tissue	
	Abs./Protein	Abs./Fwt.	Abs./Protein	Abs./Fwt.
Control	7.72	0.66	9.59	10.1
DDCA	8.86	0.71	9.50	13.2
NA	9.91	0.87	12.43	15.2

Abs./Protein = Absorption 530nm / (ug Protein / 0.1ml enzyme extract).

Abs./Fwt. = Absorption 530nm / g fresh weight extracted tissue.

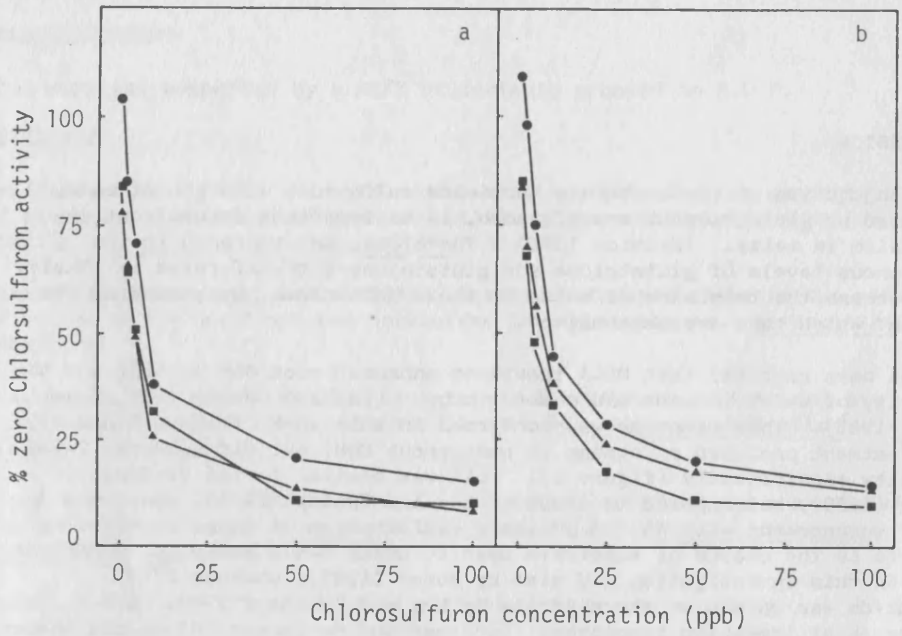

Fig. 3. AHAS / Chlorsulfuron inhibition curves with DDCA / NA pretreatments in a) shoot and b) root. (●——●) control, (■——■) DDCA and (▲——▲) NA.

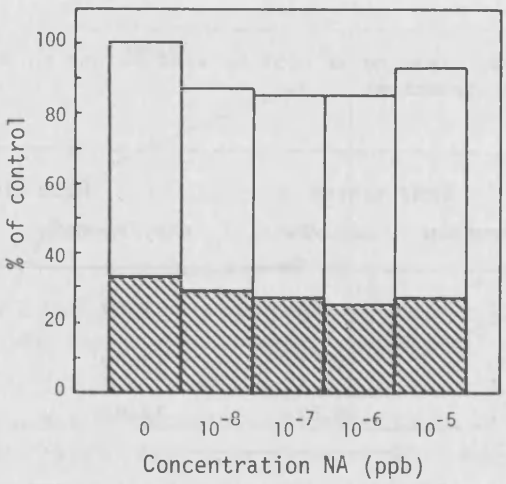

Fig. 4. Effect of NA *in vitro* upon AHAS activity ± Chlorsulfuron.
□ 0 ppb Chlorsulfuron, ▨ 10 ppb Chlorsulfuron.

DISCUSSION

The conjugation of thiocarbamate herbicide sulfoxides with glutathione, mediated by glutathione-S-transferases, is an important detoxification mechanism in maize. (Hatzios 1982). Therefore, any increase in the endogenous levels of glutathione and glutathione-S-transferases is likely to increase the tolerance of maize to these herbicides, by enhancing the rate at which they are metabolised.

It has been reported that DDCA treatment enhanced root GSH content and the activity of G-S-T in root and shoot tissue. (Lay and Casida 1976, Mozer *et al* 1983). This response was confirmed in this work. (figure 1 and 2). NA treatment produced no change in endogenous GSH, but did increase G-S-T activity significantly (figure 1). This was similar to the findings of Mozer (1983), but opposed to those of Lay and Casida (1976), who found no G-S-T enhancement with NA. A possible explaination of these conflicting reports is the choice of substrate used to assay G-S-T activity. CDNB was used in this investigation and also by Mozer (1983), whereas EPTC sulfoxide was chosen as the subtrate by Lay and Casida (1976). G-S-T occurs as at least two isoenzymes (Guddewar and Dauterman 1979), and these have been shown to have different substrate specificities (Edwards and Owen 1986). Hence NA may enhance G-S-T isoenzymes which are active with CDNB, but not EPTC sulfoxide.

The use of other chemicals which modulate GSH levels and G-S-T activity could provide a means of modifying the sensitivity of both crop and weed plants to herbicides such as the thiocarbamates, thus extending their range of use. The herbicide tridiphane which inhibits G-S-T activity (Lamoureux et al 1986) has been used to synergise the activity of EPTC in the grass weed giant foxtail (Setaria faberi) (Ezra et al 1985). Other compounds, such methionine sulphoximine, which inhibit glutathione biosynthesis (Rennenberg and Uthemann 1980) may prove of value.

The safening of maize to sulfonylurea herbicides was reported to be associated with an increased rate of metabolism via the cytochrome P-450 mixed function oxidase system (Sweetser 1985). Since both this enzyme system and G-S-T activity are enhanced by safener treatment, the possibility that AHAS activity is also enhanced should not be overlooked as a possible mechanism of action.

The results presented here (figures 3 a and b, table 1) indicate that although AHAS activity was enhanced in safened plants, the sensitivity of AHAS to Chlorsulfuron was also increased. NA was also shown to have no effect in vitro upon AHAS inhibition by chlorsulfron (figure 4) and thus would be unlikely to alter the binding of the herbicide with the enzyme.

Hence, the safening of maize to chlorsulfuron by NA and DDCA, did not appear to be explained by the changes in AHAS activity observed.

ACKNOWLEDGEMENT

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